ABSTRACT

NEURO-IMMUNE MECHANISMS IN RESPONSE TO *VENEZUELAN EQUINE ENCEPHALITIS VIRUS* INFECTION

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Venezuelan equine encephalitis virus (VEE) is an emerging pathogen with epizootics and epidemics occurring in the Western Hemisphere. Recent outbreaks in South America have caused significant morbidity and mortality among domesticated livestock and surrounding human communities. VEE pathogenesis is characterized by infection of the central nervous system (CNS) where the virus targets neurons, resulting in significant neurodegeneration. VEE encephalitis can result in profound neurological deficits or even death. Because of the devastating nature of this disease and the lack of interventional therapies, it is important to understand the intricate details of VEE neuropathogenesis in order to identify targets for treatment to effect a cure.

Inflammation has recently been implicated as a component of neurodegeneration. Inflammation in the CNS in response to acute infections is a protective mechanism that attempts to contain and clear neuro-invasive pathogens, however this upregulation of pro-inflammatory genes may be deleterious to surrounding neurons. The combined effects of direct infection and inflammation may be additive or synergistic in the amount of injury sustained in the CNS.

Glial cells are of particular importance in the CNS immune response. These resident cells of the CNS have intimate associations with neurons and regulate the CNS milieu. One type of glial cell is the astrocyte. Astrocytes are found in vast numbers in the CNS and have essential functional roles in maintaining a healthy environment for neurons. Further, astrocytes play a role in the pro-inflammatory innate immune response.

To identify the role of astrocytes in VEE infection, I characterized astrocyte susceptibility to VEE infection using an *in vitro* culture system and have further described their pro-inflammatory responses following VEE infection. Specifically, inducible nitric oxide synthase, tumor necrosis factor-alpha, and interleukin-6 are upregulated in response to VEE infection in primary astrocyte cultures as shown by reverse transcriptase-polymerase chain reaction and analyses of protein synthesis. I have also demonstrated that there were quantitative differences in the upregulation of these responses between virulent and attenuated strains of VEE.

To characterize the pro-inflammatory response *in vivo*, I measured cytokine gene expression in the CNS using a murine model of VEE infection. The cytokine responses to virulent VEE resulted in the upregulation of multiple genes important in inflammation and apoptosis. In contrast, cytokine responses in the CNS were delayed or absent following infection with attenuated VEE, depending on the specific mutant VEE strain.

Finally, CNS tissue from mice infected with VEE was double-labeled for astrocytosis and apoptosis, and stained for VEE antigen in adjacent tissue sections. Apoptosis occurs not only in areas of the brain where VEE antigen could be detected, but also in areas of acute astrogliosis, where no VEE antigen could be demonstrated. This

association of apoptosis and astrogliosis suggests that inflammation may be contributing to neuronal degeneration in response to VEE infection.

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by

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LIST OF ABBREVIATIONS

ANOVA	analysis of variance
AST	average survival time
BBB	blood-brain barrier
BDNF	brain-derived neurotrophic factor
BSA	bovine serum albumin
CNS	central nervous system
CPE	cytopathic effects
DAB	diaminobenzidine
DCS	donor calf serum
DEPC	diethylepyrocarbonate
DMEM	Dulbecco s minimum essential media
dNTP	deoxynucleotide triphosphate
EEE	*Eastern equine encephalitis virus*
ELISA	enzyme-linked immunosorbent assay
Fas L	Fas ligand
FBS	fetal bovine serum
GAPDH	glyceraldehyde-3-phosphate dehydrogenase
GDNF	glial cell line-derived neurotrophic factor
GFAP	glial fibrillary acidic protein
HRP	horseradish peroxidase
IFN	interferon
IFN-_/_	interferon-alpha/beta

IFN-_	interferon-gamma
IL-1_/_	interleukin-one alpha/beta
IL-6	interleukin-six
IL-12	interleukin-twelve
iNOS	inducible nitric oxide synthase
IRF	interferon regulatory factor
IRF-1	interferon regulatory factor-one
IRF-2	interferon regulatory factor-two
JEV	*Japanese encephalitis virus*
L-NAME	*N*-nitro-L-arginine-methyl ester
LPS	lipopolysaccharide
LSD	least statistical difference
MOI	multiplicity of infection
MTB	*Mycobacterium tuberculosis*
NGF or _NGF	nerve growth factor
NO or $^{\bullet}$NO	nitric oxide
NO^-_2	nitrite
O.D.	optical density
PBS	phosphate buffered saline
PFU	plaque forming unit
p.i.	post-infection
RPA	ribonuclease protection assay
RT-PCR	reverse transcription-polymerase chain reaction

SB	*Sindbis virus*
SEM	standard error of the mean
SCID	severe combined immune deficiency
TBE	*Tick-borne encephalitis virus*
TdT	terminal deoxynucleotidyl transferase
TNF-_	tumor necrosis factor alpha
TRAIL	TNF-related apoptosis inducing ligand
TUNEL	terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling
VEE	*Venezuelan equine encephalitis virus*
VSV	*Vesicular stomatitis virus*
WEE	*Western equine encephalitis virus*

CHAPTER 1

Introduction and Background

Arthropod-transmitted viruses (arboviruses) are members of an epidemiological class of viruses that replicate in blood-feeding arthropods and are transmitted by bite to the vertebrate host. Because of the wide distribution of arthropods, and the pathogenic viral agents they transmit, arboviruses pose a significant health threat to humans. Early attempts to categorize these diseases were based on serological groupings and led to their classification into Group A and Group B arboviruses. Group A arboviruses eventually became identified as the genus *Alphavirus* of the family *Togaviridae* (White and Fenner, 1994). Of the 27 members in the genus *Alphavirus*, 11 have been shown to cause disease in humans and 8 produce significant epidemics. During the 1930s, three distinct, but antigenically related, viruses were recovered during autopsies on dead horses. They were subsequently shown to be previously unrecognized viral agents of severe equine encephalitis (Smith *et al*., 1997). One member of this important group of mosquito-transmitted diseases is *Venezuelan equine encephalitis virus* (VEE), which was isolated in the Guajira peninsula of Venezuela in 1938 (Kubes and Rios, 1939). As had been reported for the two related equine encephalitic viruses, *Eastern* and *Western equine encephalitis viruses* (EEE and WEE, respectively), it was apparent that disease was not restricted to domesticated animals, but also caused disease in humans located in close proximity to infected livestock. Over the next 30 years, VEE outbreaks occurred at approximately 10 year intervals in several South American countries. Eventually, epizootics of VEE spread throughout the Americas reaching as far as south Texas in the 1970s, where tens of thousands of horses died and 112 confirmed or suspected cases were

reported in humans as a result of the disease spreading across the border from Mexico (Phelps, 1971, Sudia *et al*., 1975). The most recent epizootic outbreak of VEE occurred in Venezuela and Colombia during 1995, where an estimated 75,000 to 100,000 people were infected with a case fatality rate of 0.7%, mostly in small children (Weaver *et al*., 1996).

Enzootic strains of VEE are found in Venezuela and Colombia, and also in the Florida everglades (White and Fenner, 1994). They occur in forests and marshes and develop an epizootic characteristic that is promoted during wet and rainy seasons when mosquito populations explode. The primary vectors for VEE are *Culex* or *Aedes* mosquito species that circulate among vertebrate hosts, specifically rodent or birds (Nathanson, 1996, Weaver *et al*., 1996). Indeed, migratory birds have been suspected in the widespread distribution of VEE during epizootic outbreaks (Phelps, 1971). The natural life cycle is thought to be transmission of the virus from its reservoir, infected rodents and birds, where VEE can replicated to high titers. The mosquito transmits VEE during feeding on a susceptible host via saliva where the virus is transported to lymphoid tissue and begins its replication cycle. VEE can also undergo an amplifying cycle where domesticated animals such as horses or rabbits are infected. In these amplifying hosts, VEE replicates to high titers, and because of their close proximity with humans, the virus can be transmitted to the human population. VEE can replicate to high serum titers in humans, but are incidental or "dead-end" hosts and do not serve as a substantial reservoir in disease transmission (Nathanson, 1996).

All alphaviruses share structural properties and common antigenic determinants, found on the nucleocapsid protein, named C. The six antigenic subsets of alphaviruses

are further distinguished by unique antigenic determinants found on the envelope glycoproteins, *E1* and *E2* (White and Fenner, 1994). The VEE virions have a spherical shape with a diameter of 70 nm, and are enveloped in a lipid bilayer derived from the host cell plasma membrane (Schlesinger and Schlesinger, 1996). This spherical, icosahedral structure contains a linear, message-sensed single stranded RNA genome of 11-12 kilobases. The organization of the alphavirus genome is arranged into two distinct subsets: 1) genes that encode nonstructural proteins located at the 5′ end of the genome required for transcription and replication of the viral RNA and 2) genes for structural proteins located at the 3′ end encoding the capsid and glycoproteins. The replication cycle starts by virions being taken up into coated vesicles via receptor-mediated endocytosis. Virions are uncoated when phagolysosomes reach a low internal pH. As an RNA virus, VEE replication occurs in the cytoplasm of the infected host cell, where full-length and subgenomic RNA transcripts are synthesized and translated into proteins. Some translated proteins require post-translational cleavage to produce mature proteins (White and Fenner, 1994, Schlesinger and Schlesinger, 1996). Genomic RNA is then packaged in the capsid nucleoprotein and migrates to the plasma membranes, whereas two of the structural proteins, *E1* and *E2*, are glycosylated in the endoplasmic reticulum and the Golgi apparatus, and then inserted into the host plasma membrane. The final assembly of mature virions occurs as the nucleocapsid containing the genomic RNA buds from the host cell plasma membrane in sites containing the *E1* and *E2* glycoprotein spikes.

The pathogenesis of VEE in the vertebrate host is described as a biphasic infection of the periphery and the central nervous system (CNS). After the infected-

mosquito introduces VEE into the susceptible host while feeding from a capillary, the virus begins to replicate in the vascular endothelium and in blood monocytes and macrophages (White and Fenner, 1994, Schlesinger and Schlesinger, 1996). Alternatively, it has been demonstrated that initial targets for VEE infection are specialized antigen presenting cells, the Langerhan cells, in the skin (MacDonald and Johnston, 2000). These VEE-infected dendritic cells then migrate to the draining lymph node where VEE replicates in the lymphoid tissue and spleen, causing a marked destruction of lymphocytes and extensive follicular necrosis (Jackson *et al*., 1991), producing a viremia. Viremia leads to systemic dissemination of the virus with infection of multiple organ systems including the small intestine, liver, pancreas, thymus, heart, lungs, kidneys, adrenal glands, muscles, joints, bone marrow, salivary glands, and the eyes (Tasker *et al*., 1962, Jackson *et al*., 1991, Grieder *et al*., 1995). This systemic infection manifests in clinical symptoms of fever, headache, muscle and joint pain. After an initial course of 3 - 5 days, VEE is cleared from the periphery, then establishes an infection in the CNS. Suspected routes of entry for VEE into the CNS include the olfactory neuroepithelium (Charles *et al*., 1995, Davis *et al*., 1994, Ryzhikov *et al*., 1995, Vogel *et al*., 1996), other cranial nerves, such as the trigeminal (Steele *et al*., 1998) or optic nerves (Grieder *et al*., 1995), endothelium of the CNS vasculature (White and Fenner, 1994), circumventricular organs of the CNS where the blood-brain barrier (BBB) is fenestrated, or trafficking of infected immune cells from the periphery into the CNS (Charles *et al*., 1995). The consequence of VEE infection in the CNS is an acute meningoencephalitis, with direct infection of neurons and a panencephalopathic inflammatory response consisting of infiltrating immune cells from the periphery,

vasculitis, gliosis, necrosis, apoptosis, and cerebral edema (Garcia-Tamayo *et al*., 1979, de la Monte *et al*., 1985, Grieder *et al*., 1995, Jackson *et al*., 1991, Jackson and Rossiter, 1997, Schoneboom *et al*., 2000a). Clinical signs of VEE infection usually persist for 5 - 10 days. Encephalitis develops in approximately 3% of cases, with a mortality rate as high as 0.5%, however the mortality rate in young children is approximately 10-fold higher (Brooks *et al*., 1998). Survivors from VEE encephalitis are often left with permanent neurological sequelae such as blindness, deafness, cognitive dysfunction, paralysis, or seizure disorders (White and Fenner, 1994). VEE also poses a significant threat to the fetus; pregnant women infected with VEE frequently spontaneously abort or the pregnancy ends in an intrauterine death (Casamassima *et al*., 1987, Weaver *et al*., 1996). Currently, there is no specific treatment for VEE infection except supportive measures such as analgesics for aches and fever control, fluid and electrolyte maintenance, seizure management, and ventilatory support (Ray, 1994, Sanford, 1994, Eitzen *et al*., 1998).

The best line of defense against VEE infection is prevention. At present, these measures include vector control consisting of aerosolization of pesticides, behavior modification including restriction of outdoor activities or wearing protective clothing, and elimination of mosquito breeding grounds such as stagnant water pools. There is a vaccine available that is effective in horses and is used experimentally in humans for protection of laboratory personnel who are exposed while working with live, virulent VEE. This live attenuated strain of VEE, which was developed as a vaccine, was isolated by 83 passages of a virulent parental Trinidad donkey strain of VEE in guinea pig heart cells and is identified by the abbreviation TC-83 (Strauss and Strauss, 1994). This

vaccine is not ideal, however, because of a high failure of seroconversion (approximately 20% of vaccinated volunteers fail to seroconvert), and due to undesirable side effects including flu-like symptoms and even encephalitis (Pittman *et al*., 1996, Burke *et al*., 1977, Casamassima *et al*., 1987). Nonetheless, this vaccine has been a valuable tool in VEE research. VEE poses substantial risk to researchers and laboratory personnel as VEE-infected animals and virus stocks of high titer concentrations can aerosolize particles containing VEE. Unprotected personnel and animals in confined areas with aerosolized VEE particles can become exposed and infected via inhalation (Lennette and Koprowski, 1943, Shubladze, 1959, Slepushkin, 1959).

One historical, and now renewed focus of VEE is its suitability for weaponization. The United States has a history of weaponizing VEE in the 1950s and 1960s prior to the termination of the U.S. offensive biowarfare program (Eitzen *et al*., 1998). Other countries have also been, or are suspected of, utilizing VEE in their biowarfare programs (Vogel *et al*., 1997). In addition, factions not under the auspices of a recognized government, could easily employ VEE in a bio-terrorist attack (Hursh, 1996). Some of the qualities of VEE that make it suitable as a bio-weapon include: 1) it is readily cultured in egg embryos or other culture systems that permit production of VEE virus in vast quantities, 2) infection can occur through the respiratory route after aerosolization, 3) casualties have been estimated at 50% in an exposed, unimmunized population, 4) immunization of at-risk populations with the experimental vaccine TC-83 is the only preventative measure available, yet seroconversion with TC-83 is only 80% rendering a substantial number of people unprotected, 5) a rapid decay rate occurs after deployment

that would render the area suitable for occupation by a hostile force, and, 6) the capability to inflict substantial psychological damage.

Significant advances in VEE research have been achieved since the molecular cloning of VEE (Davis *et al*., 1989). The advantage of studying the pathogenesis of VEE using virus derived from a molecular clone is that the virus population is homogenous. This is in contrast to "biological" RNA viruses, which are heterogeneous in their genetic makeup due to natural mutations. Further, well-characterized VEE attenuated phenotypes have been developed by site-directed mutagenesis (Davis *et al*., 1991), which permit evaluations of genotypic and phenotypic alterations *in vitro* (Grieder and Nguyen, 1996, Schoneboom *et al*., 1999) and *in vivo* (Davis *et al*., 1991, Davis *et al*., 1989, Grieder *et al*., 1995, Grieder *et al*., 1997, Grieder and Vogel, 1999, Schoneboom *et al*., 2000b). The VEE clones used in the following experiments differ by single, specifically selected nucleotides from the virulent parent, resulting in viruses that differ by only single amino acids (Davis *et al*., 1991, Grieder *et al*., 1995). V3000 is the virulent parental VEE clone derived from the Trinidad donkey strain that results in a 100% mortality rate in the mouse model (Grieder *et al*., 1995, Grieder *et al*., 1997). V3010 is a neuro-invasive, attenuated virus with a mutation at glycoprotein *E2* position 76 replacing a glutamic acid in V3000 for lysine in the mutant V3010. This mutation reduces the mortality rate from 100% with V3000 to 10% with V3010 in the C57BL/6J inbred mouse strain following the peripheral rear footpad injection. V3034, also neuro-invasive, has a mutation at glycoprotein position *E1*-272; threonine substitutes for alanine in the V3034 strain resulting in an attenuated virus with a mortality rate of 20% when injected by the same peripheral footpad route.

The classic studies of VEE pathogenesis have been performed in rodent models, specifically mice, rats, and hamsters (Austin and Scherer, 1971, Davis *et al*., 1994, Grieder *et al*., 1995, Gorelkin, 1973, Gorelkin and Jahrling, 1975, Jackson *et al*., 1991, Jahrling and Scherer, 1973, Jahrling *et al*., 1978, Jahrling and Stephenson, 1984, Neufeld *et al*., 1978, Walker *et al*., 1976). These studies were based on the hypothesis that rodents are the natural vertebrate reservoirs of VEE in the environment and mimic the course of infection in humans and equines. These studies were important in characterizing infectivity and histopathology of VEE infection and demonstrated unique differences between mice and hamsters (Jackson *et al*., 1991). Adult hamsters, infected with the virulent Trinidad donkey strain of VEE usually did not survive past day 3 post-infection (p.i.). Most of the pathologic changes in hamsters were noted in extraneural tissues, particularly the Peyer's patches of the small intestine, and died before CNS infection could be established. In contrast, adult mice infected with VEE cleared the systemic infection, and developed signs of encephalomyelitis including hindlimb paralysis. These VEE-infected mice demonstrated significant VEE antigen in the CNS, as well as neuronal degeneration associated with inflammation, and died on day 8 p.i. Because of these significant differences between the two rodent models, mice have been the preferred model in recent years for VEE research as the clinical course of the VEE infection and the development of encephalitis mimics disease progression in humans and equines.

This well-characterized model of VEE infection in mice continues to be extensively used in research and serves as a model system for other types of human encephalitides that are endemic to North America, such as EEE, WEE, and the recently

described West Nile-like encephalitis virus (MMWR, 1999a). A recent emphasis has been the characterization of the innate immune response to VEE in the periphery (Grieder *et al*., 1997) and in the CNS (Grieder and Vogel, 1999, Schoneboom *et al*., 2000a, Schoneboom *et al*., 2000b). The goal of characterizing the innate immune response to VEE infection is to understand the contributions of inflammation to the development of CNS pathology and to identify those responses that are essential for host survival. The ultimate aim of this line of research is to manipulate the innate immune response to maximize beneficial and minimize detrimental immune responses.

Early work describing the neuropathogenesis of VEE infection in mice identified areas of neurodegeneration associated with direct infection of neurons with VEE, but also marked inflammatory infiltrates consisting of polymorphonuclear leukocytes, lymphocytes, and macrophages in the perivasculature, extending into the CNS parenchyma and meninges (Jackson *et al*., 1991). This study also identified VEE antigen in the ventral horn of the spinal cord in mice and attributed the common finding of hindlimb paralysis to this specific area of neurodegeneration. This finding of an acute inflammatory response in the CNS in experimental animals is further substantiated by autopsy histopathology from documented VEE infections in humans where widespread perivascular cuffing and necrotizing vasculitis were evident (de la Monte *et al*., 1985).

Studies describing neurodegeneration in the CNS of acute VEE infection characterized the type of neuronal cell death as necrosis (Jackson *et al*., 1991, Steele *et al*., 1998, de la Monte *et al*., 1985) based on morphologic features. But as our appreciation of differential types of cell death developed, another component of VEE-induced neurodegeneration began to emerge: apoptosis. Apoptosis, a type of

programmed cell death, is a physiologic process in response to diverse stimuli found during embryonic development, maturation and differentiation of the immune system, and in normal tissue turnover (Lo *et al*., 1995, Buja *et al*., 1993). It has been hypothesized that apoptosis is a protective host response for elimination of virus-infected cells or as a strategy to prevent viral spread (Vaux and Hacker, 1995). However, apoptosis can also be a pathologic process. In the CNS, apoptosis can have devastating consequences due to the limited regenerative ability of neurons. The induction of certain pro-inflammatory cytokines, such as tumor necrosis factor alpha (TNF-α), has also been implicated with the initiation of apoptosis (Garside *et al*., 1996). These pro-inflammatory cytokines are produced by macrophages and T cells in response to infection (Benjamini *et al*., 1996). Because of the pro-inflammatory characteristics of VEE infection in the CNS, and the discovery that apoptosis is caused by other alphaviruses in the CNS (Griffin *et al*., 1994, Lewis *et al*., 1996), investigations into mechanisms of neuronal cell death in VEE infection also found apoptosis as a component of neuronal injury (Jackson and Rossiter, 1997).

Cellular defenses are an integral component of innate immunity against viral pathogens in the CNS. These cellular defenses consist of cells from the periphery and resident glial cells. Although the CNS has been described as "immunologically privileged", because of its structural and functional isolation from the peripheral immune system due to the blood-brain barrier (BBB), it is now known that immune cells from the systemic circulation can have access to the CNS. Several cell types from the periphery are involved in CNS surveillance including polymorphonuclear leukocytes, natural killer cells, macrophages, and cytotoxic T cells. Their functions in the innate immune response

include antigen presentation, and the production of free radicals and proteins that are toxic to viruses or infected cells (Benjamini *et al*., 1996). Immune cell migration into the CNS from the periphery leads to inflammation, a complex process characterized by the release of soluble regulatory molecules, specifically, reactive oxygen and nitrogen species, proteases, chemokines, and cytokines. The result of an inflammatory response in the CNS is a change in the permeability of the capillary endothelium and is characterized by the migration of peripheral immune cells into the CNS parenchyma. This inflammatory response from the periphery can be beneficial with ultimate clearance of the pathogen, as well as detrimental, with the destruction of surrounding tissue, including neurons.

Glial cells also participate in the cellular defenses of the CNS to viral pathogens. The two types of glial cells with known innate immune functions are microglia and astrocytes. Microglia are the resident macrophages of the CNS, thought to arise from a common embryologic origin, the mesoderm (Cuadros and Navascues, 1998, Wozniak, 1998). Astrocytes, in addition to structural and homeostatic support of the CNS, also participate in innate immune responses of the CNS to viral infections. Astrocytes, in partnership with microglia, respond to brain infection or injury by coordinating the immune response (Benveniste, 1992, Mucke and Eddleston, 1993). Following a CNS insult, both astrocytes and microglia transition quickly from a resting to an activated state. Activation is a graded, reversible process during which glial cells proliferate, become phagocytic, secrete cytokines, chemokines, and growth factors, present antigen, and finally become cytotoxic, by producing superoxide and nitric oxide ($^{\bullet}$NO) (Aloisi *et al*., 1992, Altman, 1994, Bechmann and Nitsch, 1997, Grau *et al*., 1997, Hellendall and

Ting, 1997, Neumann *et al*., 1996, Nikcevich *et al*., 1997, Schiffer *et al*., 1993, Streit and Kincaid-Colton, 1995, Zielasek and Hartung, 1996, Zielasek *et al*., 1996). Astrocytes and microglia may also participate in the anti-viral immune response by presenting viral antigen in context with MHC to activated T cells (Griffin, 1995) and producing pro-inflammatory cytokines in response to virus infections *in vitro* (Lieberman *et al*., 1989, Brodie *et al*., 1997) and *in vivo* (Sun *et al*., 1995).

This concert of innate immune events mediated by peripheral and CNS immune cells in response to viral infection is composed of multiple, pro-inflammatory signals and results in a complex system of interacting cytokines and other mediators. Several studies that have characterized these pro-inflammatory responses in the CNS following neurotropic viral infection have demonstrated an upregulation of multiple cytokine genes. Specifically, experiments using Borna disease virus found that TNF-α, IL-1α/β, and IL-6 mRNA were up-regulated, and that these inflammatory mediators were produced by resident cells in the CNS, such as astrocytes and microglia (Sauder and de la Torre, 1999). Cytokine profiles of virulent and attenuated strains of the mouse hepatitis virus JHM also found an early up-regulation of TNF-α in virulent JMH-infected mice, transient increases in mRNA for IL-12, IL-1α /β, IL-6, and iNOS, and that these responses were different depending on the virus phenotype (Parra *et al*., 1997). Furthermore, chronic infection of mice with JHM resulted in an up-regulation of a similar panel of pro-inflammatory mediators (TNF-α, IL-1β, IL-6, and iNOS) in astrocytes in the spinal cord. Finally, astrogliosis was not limited to areas in the CNS that were positive for viral antigen, but was also found in areas without JHM infection (Sun *et al*., 1995).

Studies of innate immune responses in the CNS to VEE infection have shed some light on the balance between host protection and neurotoxicity. Specifically, studies investigating the role of interferon regulatory factors 1 and 2 (IRF-1 and IRF-2), transcription factors important in the upregulation of the type I interferons (IFN-α/β) (Kimura *et al*., 1994) and $^{\bullet}$NO (Kamijo *et al*., 1994), found that IRF-1 and IRF-2 knockout mice were more susceptible to VEE infection as compared to control mice (Grieder and Vogel, 1999). Histopathologic analysis of VEE-infected IRF-2 knockout mice showed a lack of immune cell infiltration from the periphery into the CNS parenchyma and an increase in gliosis resulting in marked neurodegeneration, suggesting that certain components of the inflammatory response are indeed essential in host protection against VEE. Even more dramatic were mortality rates among IFN-α/β receptor knockout mice *vs*. control mice. IFN-α/β knockout mice had a significant decrease in survival times with accelerated virus penetration into the CNS (Schoneboom *et al*., 2000b). Furthermore, attenuated phenotypes of VEE were 100% virulent in IFN-α/β receptor knockout mice. Therefore, IFN-α/β appears to play a critical role in host protection against VEE, although it is not sufficient for complete protection against virulent VEE infection. To contrast these previous studies of innate immune responses in host protection, studies comparing severe combined immune deficiency (SCID) mice and immunologically normal mice (Charles *et al*., 2000) found longer survival times in SCID mice as compared to immunocompetent control mice and distinctly different histopathology in the CNS. These findings suggest that certain components of the innate immune response contribute to the neuropathogenesis of VEE infection.

The aim of this research project was to explore pro-inflammatory responses in the CNS in response to VEE infection, with specific emphasis on the role of astrocytes. The hypothesis that was tested is that VEE induces altered production of cytokines and neuronal growth factors in astrocytes that might influence neuronal degeneration. The experimental models used to test this hypothesis include: 1) the neuro-invasive, molecularly cloned VEE phenotypes, virulent V3000, and the attenuated mutants, V3010 and V3034, as well as 2) *in vitro* and *in vivo* models. Together, this model system was used to characterize the pattern of pro-inflammatory responses at the transcriptional and translational level, and to investigate whether VEE infection, and its associated inflammatory response, induce apoptosis in astrocytes.

CHAPTER 2

ASTROCYTES AS TARGETS FOR VENEZUELAN EQUINE ENCEPHALITIS VIRUS INFECTION

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Abstract

Venezuelan equine encephalitis virus (VEE) produces an acute infection in humans and induces a well-characterized cytopathic effect in neurons of the central nervous system (CNS). However, little is known about the role of glial cells in response to VEE infection of the CNS. Our results demonstrate that VEE is capable of a productive infection in primary astrocyte cultures and that this infection is cytotoxic. Further, there were significant differences in the growth kinetics comparing virulent and attenuated strains of VEE. Additionally, VEE infection of astrocyte cultures induced gene expression of two neuro-immune modulators, tumor necrosis factor-alpha (TNF-α) and inducible nitric oxide synthase (iNOS). Assays for TNF-α protein and nitric oxide (NO) demonstrated high levels of TNF-α protein and low levels of NO in response to VEE infection of astrocytes. These observations suggest an important role of astrocytes in this virus-induced encephalitis, and that interactions between astrocytes, other glial cells, and neurons may be important in VEE pathogenesis. Such interactions, which could impact neuronal survival, may include loss of functional changes in astrocytes or, alternatively, their production of neurotoxic molecules.

Introduction

Glial cells play a central role in neuronal function and viability. Astrocytes, the predominant glial cell type in the central nervous system (CNS), outnumber neurons by a ratio of 8:1 and carry out critical functions in normal CNS physiology including neuronal guidance during development (Juliano *et al*., 1996), structural processes for the blood-brain barrier (Janzer and Raff, 1987), buffering of potassium ions, removal of neurotransmitters, specifically glutamate (Keyser and Pellmar, 1994, Marrero and Orkand, 1996, Orkland, 1994, Vernadakis, 1996), and synthesis of essential neurotrophic factors (Gray and Patel, 1992, Muller *et al*., 1984, Schmalenbach and Muller, 1993, Wilkin *et al*., 1990, Yoshida and Gage, 1991). Dysfunction of the CNS can result from trauma, hypoxia, toxic chemicals, radiation, neurodegenerative diseases such as Parkinson's or Alzheimer's diseases, and certain neurotropic infections including virus infections. Regardless of the type of insult, neuronal degeneration appears to be due to a final common pathway (Dugan and Choi, 1994) that includes glial cell activation or dysfunction (Mucke and Eddleston, 1993). This results in loss of trophic support, production of free radicals, or excitotoxicity.

Venezuelan equine encephalitis virus (VEE) is a positive-sense RNA virus indigenous to Central, South and parts of North America. A member of the Togaviridae family, this mosquito-transmitted virus causes encephalitis in horses, but is also capable of causing disease in humans. The most recent outbreak of the disease occurred in 1995 where over 12,000 cases were reported in South America (MMWR, 1995). The characteristic clinical features of this infectious encephalitic disease include headache, fever, chills, skin rash, and malaise. Clinical signs usually persist for 5-10 days, and

encephalitis develops in approximately 0.4% of cases, however, children are particularly vulnerable as their occurrence of encephalitis is approximately 4% (de la Monte *et al*., 1985, Kissling and Chamberlain, 1967).

The pathogenesis of VEE has been described as a primary infection of lymphocytes and neurons (Johnson *et al*., 1997, Jackson *et al*., 1991, Jackson and Rossiter, 1997, Grieder *et al*., 1995, Grieder and Nguyen, 1996, Grieder *et al*., 1997). While it has been established that neurons in the CNS undergo degeneration following VEE infection (Grieder *et al*., 1995, Jackson *et al*., 1991, Jackson and Rossiter, 1997, Charles *et al*., 1995), the role of glial cells during the acute stage of VEE infection is unclear. Astrocytes are targets for other viral infections including *JC virus* (Aksamit *et al*., 1986), *Theiler's virus* (Aubert *et al*., 1987), *Visna virus* (Stowring *et al*., 1985), *Borna virus* (Carbone *et al*., 1991), as well as *Human Immunodeficiency virus* type-1 (Tornatore *et al*., 1994). However, these viruses induce slow, progressive diseases of the CNS, which differ from VEE in that VEE causes an acute, rapidly progressing disease. Understanding the responses of astrocytes in an acute infection and how these responses differ from slowly progressing infections is necessary for understanding acute inflammatory responses in the CNS.

Recently, an understanding of the unique immune system of the CNS has emerged. Previously, the CNS was described as "immunologically privileged", due to the isolation of the CNS from immune surveillance by circulating lymphocytes because of the blood-brain barrier, and the absence of a lymphatic drainage system. Recognizing these differences in CNS immune system structure and function, a relatively new area of research has developed to characterize how the CNS responds to infections and injury. It

is now understood that glial cells, specifically astrocytes and microglia, play a critical role in orchestrating the immune response of the CNS. Besides their physiologic role in CNS homeostasis, astrocytes can function as antigen presenting cells in association with major histocompatibility complex class I and class II molecules on their surface (Mucke and Eddleston, 1993, Neumann *et al*., 1996, Nikcevich *et al*., 1997), secrete cytokines and growth factors (Chung and Benveniste, 1990, Benveniste, 1992, Brodie *et al*., 1997, Clatterbuck *et al*., 1996, Lieberman *et al*., 1989, Mendez E, 1997), phagocytose debris (Bechmann and Nitsch, 1997), and produce reactive oxygen and nitrogen intermediates (Banati *et al*., 1993, Brodie *et al*., 1997, Chao *et al*., 1996).

Our experiments characterize VEE infection of astrocytes *in vitro* and describe VEE replication kinetics and VEE induction of astrocyte cell death. Experiments were also conducted to determine if astrocytes could respond to VEE infection by the upregulation of certain pro-inflammatory molecules at the level of gene transcription and translation. Specifically, tumor necrosis factor-α (TNF-α) and inducible nitric oxide synthase (iNOS) were assessed by means of reverse transcriptase polymerase chain reaction (RT-PCR) and Southern blot analysis because of the documented production of these two molecules in response to various stimuli including interferon-gamma (IFN-γ) or lipopolysaccharide (LPS) (Lowenstein *et al*., 1993, Xie *et al*., 1993) and certain neurotropic viruses (Lieberman *et al*., 1989). Furthermore, TNF-α protein secretion into the culture supernatant, as well as nitrite (NO^-_2), the stable oxidation product of nitric oxide (NO), were assessed by quantitative assays. These experiments form the basis for understanding the role of glial cells in early neuro-immune responses against this acute viral infection and the potential impact on neurons of the CNS.

Materials and Methods

Virus. Two molecularly cloned VEE, virulent V3000 and attenuated V3010, were used (Davis *et al*., 1991, Grieder *et al*., 1995). The virulent V3000 and neuro-invasive, attenuated clone, V3010 differ in their genotype by one single nucleotide resulting in a single amino acid change at glycoprotein E2 position 76. This nucleotide mutation replaces a glutamic acid in V3000 for lysine in V3010. Virulent V3000 is 100% fatal in mice regardless of route of injection. Attenuated V3010 is nonlethal when injected peripherally, and only has a 20% mortality rate when injected intracerebrally (Grieder *et al*., 1995). Molecularly cloned virus stocks were stored at -80°C and all experiments were conducted in a biosafety level 3 laboratory.

Primary Astrocyte Cultures. Primary astrocyte cultures were established as previously described (McCarthy and de Vellis, 1980). Briefly, cerebrums were removed from two-day-old Sprague-Dawley rats using sterile technique. Meninges were stripped by microscopic dissection and cerebral hemispheres were dissociated by suction pipetting followed by centrifugation at 1000 rpms for 10 minutes. The supernatant was removed and the cell pellet was resuspended in cell culture medium. This suspension was then triturated sequentially through 18 gauge and 22 gauge needles, and cells were plated in Dulbecco's minimum essential media (DMEM) supplemented with 10% non-heat inactivated fetal bovine serum (FBS), 1% L-glutamine, and 25 μg/ml gentamycin. After the establishment of a confluent monolayer (between 10-14 days), adherent microglia were removed by rotary shaker. The resulting cultures were characterized as astrocytes by positive immunofluorescent staining for glial fibrillary acidic protein (GFAP; Sigma, St. Louis, MO), an intermediate filament which is expressed by astrocytes. The

homogeneity of these astrocytes was determined by immunostaining identical cultures with a microglia specific cell-surface marker antibody OX42 (Serotec/Harlan; Indianapolis, IN) and an oligodendrocyte specific antibody antiGal-C (Boehringer, Germany). The astrocyte primary cell cultures were determined to be greater than 95 % homogenous by three independent samplings of GFAP stained cell cultures, and comparing those cell counts to phase contrast light microscopy. Contaminating microglia and oligodendrocytes were observed only on rare occasions.

Immunoperoxidase Staining of VEE-infected Astrocytes. Astrocyte cultures were passaged one time and plated into 16-well tissue culture chamber slides (Nunc Inc., Naperville, IL) and incubated for 48 hours at 37°C, 5% CO_2. Cell counts at this time were determined to be 1.5 x 10^5 cells per well. Monolayers were infected with VEE at a multiplicity of infection (MOI) of 1.0. Mock controls were inoculated with phosphate buffered saline (PBS) only. Following 1, 6, 12, 18, 24, or 48 hours infection the cells were fixed in an ice cold 1:1 mixture of methanol and acetone for 5 minutes and allowed to air dry. Slides were treated with a 1:400 dilution of polyclonal rabbit anti-VEE serum (kindly provided by Drs. George Ludwig and Jonathan Smith, USAMRIID, Ft. Detrick, MD) and stained using an avidin-biotin-conjugated peroxidase staining kit (Vectastain ABC Kit, Burlingame, CA) with diaminobenzidine (DAB) used as the chromagen resulting in brown staining (Grieder and Nguyen, 1996). Cells were counter-stained with methylene blue to visualize nuclear structures. Controls for non-specific staining included stained mock-infected cells and infected cells treated with unimmunized rabbit serum incubated at the same dilution as the anti-VEE rabbit serum.

Virus Growth Kinetics. Astrocytes were passaged one time and plated into 60 mm cell culture plates (Corning, NY). Cultures were incubated for 5 days at 37°C, 5% CO_2 until 90% confluent. Cell counts at this time were determined to be 1.5 x 10^6 per 60 mm cell culture plate. Triplicate astrocyte cell cultures were then infected with virulent V3000 or the attenuated V3010 virus at an MOI of 1.0 and incubated for one hour at 37°C, 5% CO_2. Astrocytes were washed three times with PBS containing 0.1% donor calf serum (DCS) and 3 milliliters (mls) of media was replaced. The initial samples were collected at this time, and subsequently at 6, 12, 24, 36, 48, and 72 hours p.i. Supernatant samples containing virus were immediately frozen at -80°C. Virus titers were determined as previously described by plaque assays on BHK-21 cells (American Type Culture Collection, Rockville, MD) (Scherer *et al*., 1971, Grieder and Nguyen, 1996). Virus titers were calculated as plaque forming units (PFUs) per ml of supernatant.

Astrocyte Viability. Astrocytes were passaged one time, plated into 6-well culture plates (Corning, NY) and incubated at 37°C, 5% CO_2 for four days. Cell counts were determined to be 3 x 10^5 cells per well. Medium was removed and three independent wells of astrocytes were infected with V3000 or V3010 virus at an MOI of 1.0 and incubated at 37°C, 5% CO_2 for one hour. Mock-infected controls were treated with PBS only for one hour. Infected and control astrocytes were harvested at 6, 12, 24, 36, 48, and 72 hours p.i. Briefly, the astrocyte medium was removed and the monolayers were trypsinized. Trypsin action was neutralized with warm astrocyte medium containing 10% FBS and triturated to lift the monolayer. Cells were stained with Trypan Blue (Life Technologies, Inc., Grand Island, NY), counted by hemocytometer, and mean percentage (±SEM) of living cells calculated.

Extraction of Astrocyte mRNA. Astrocytes were passaged one time and plated into 6-well cultures plates and incubated at 37°C, 5% CO_2 for two days. Cell counts were determined to be 1 x 10^6 cells per well. Medium was removed and wells were assigned to one of the following groups: 1) uninfected PBS control; 2) a combination of recombinant rat IFN-γ (100 U/ml; Genzyme Corp, Cambridge, MA) and LPS stimulated (1μg/ml equals 200 U/ml; protein free *E.coli* K235 LPS extracted by the method of McIntire *et al*., 1967; kindly provided by Dr. Stefanie Vogel, USUHS, Bethesda, MD); and 3) infected with V3000 or V3010 at an MOI of 1.0 and incubated for one hour. Total cellular RNA was harvested from three independent samples per treatment group at 2, 6, 12, 24, and 48 hours p.i. using RNAzol™ B (Tel-Test, Inc., Friendswood, TX) as previously described (Chomczynski and Sacchi, 1987). RNA was extracted with chloroform (Sigma, St. Louis, MO), precipitated with isopropanol (Sigma, St. Louis, MO), and diluted in diethylepyrocarbonate (DEPC)-treated water (Quality Biologicals, Inc., Gaithersburg, MD). The concentration of RNA in each sample was determined using a spectrophotometer (Beckman Instruments, Inc., Columbia, MD). Specimens were stored at -80°C until processing by RT-PCR.

RT-PCR Detection of mRNA. cDNA synthesis was performed by reverse transcription in a reaction volume of 25μl using: 1) 1μg of RNA; 2) random hexamer oligonucleotides at a concentration of 0.5U; 3) 0.25mM mix of all four deoxynucleotide triphosphates (dNTPs); 4) 1X reverse transcriptase buffer (50mM Tris-HCL, pH 8.3, 75mM KCL, 3mM $MgCl_2$); 8mM DTT; and 200U Moloney Murine Leukemia virus (MMLV) reverse transcriptase (GIBCO, Gaithersburg, MD). The reaction mixture was incubated at 37°C for 60 minutes, heated to 90°C for 5 minutes to denature the enzyme

and cooled on ice before storage at -20°C. The final reaction volume was diluted with an addition 175µl of DEPC treated water.

Amplification of cDNA was accomplished using gene-specific sense and antisense oligonucleotide primers for rat glyceraldehyde-3-phosphate dehydrogenase (GAPDH), TNF-α, and iNOS (Table 1). Products amplified from cDNA could be distinguished from genomic DNA because the primers were designed to span at least one intron. To each PCR reaction the following components were added: 1) 1.0mM dNTP mix; 2) 1X PCR buffer (50mM KCL, 10mM Tris-HCL, 3mM $MgCl_2$); 3) 10µl of cDNA; 4) 0.2µM sense and antisense primers; 5) and 1U Taq polymerase (Promega, Madison, WI). PCR reaction mixture was then amplified using an automated PCR thermocycler (Perkin-Elmer, Norwalk, CT). 10 µl of amplified PCR products along with 2 µl of gel loading buffer were added to each well in a 1.5% agarose gel and electrophoresed at 90 volts for 60 minutes in 1X Tris buffer. After electrophoresis, the gels were denatured, neutralized and transferred to Hybond N^+ membranes (Amersham Life Science, Arlington Heights, IL) using 10X SSC by standard capillary Southern blotting techniques (Southern, 1975). DNA was then cross-linked to the membrane by exposure to UV light for 2 minutes and baked at 80ºC in a vacuum oven. Subsequent visualization of specific DNA bands on the blots was conducted using fluorescein labeled-oligonucleotide probes complimentary to the PCR products and detected using the enhanced chemical luminescence technique (ECL™) (Amersham LifeScience, Buckinghamshire, England). Light output was detected on Hyperfilm™ ECL™ film (Amersham LifeScience, Buckinghamshire, England), that was then scanned into a digital image. Blots were quantified by obtaining pixel densities using Scion Image software for Windows (Scion Corporation, Frederick,

MD) and normalizing relative changes in gene expression of each treatment group at each time point to their matched untreated controls. GAPDH signals were consistent for all experimental treatment groups. It is important to note that this procedure only allows for relative quantitation and comparisons of relationships among treatment groups and does not provide direct comparisons with exact levels (i.e., units) of mRNA among treatment groups (Wynn *et al*., 1993).

TNF-α Immunoassay. TNF-α protein in primary astrocyte cultures supernatant was measured using the Quantikine® M rat TNF-α kit (R&D Systems, Minneapolis, MN) as per manufacturer's instructions. Briefly, astrocyte cultures plated at 1 x 10^6 cells/well were infected with one of the molecularly cloned VEE strains at an MOI of 1.0 or treated with IFN-γ/LPS. Untreated astrocytes were used as a negative control. Supernatants were harvested from three independent wells for each treatment group at 2, 6, 12, 24, and 48 hours p.i., and media replaced after each sampling. All samples were stored at 4°C and diluted 1:2 with the calibrator diluent before assaying to bring TNF-α levels within the range of the standards. Standards, controls, and samples were assayed in duplicate at a wavelength of 450 nm and 550 nm for wavelength correction. Optical density (O.D.) was determined as the change in O.D. between the two wavelengths and intensity of the color reaction product was quantitated on a microplate reader (ELx800, Biotek Instruments, Inc., Winooski, VT) using Kineticalc software for Windows® (Version 1.5, Biotek Instruments, Inc., Winooski, VT). A range of TNF-α dilutions was used to generate a standard curve to determine TNF-α concentrations in the sample supernatant.

Nitrite Assay Procedure. The amount of nitric oxide produced by astrocytes was determined by assaying its stable oxidation product, NO^-_2 (nitrite) (Green *et al*.,

1982). Briefly, equal volumes (100µl) of sample and Griess reagent (1% sulfanilamide, 0.1% *N*-1-naphthyethylenediamine dihydrochloride in 2.5% H_3PO_4) (Sigma, St. Louis, MO) were mixed in a 96-well plate. The optical density of the color reaction product from three independent samples was measured at 540 nm with a microplate reader (ELx800, Biotek Instruments, Winooski, VT). A range of sodium nitrite dilutions was used to generate a standard curve. Values were quantified using Kineticalc software for Windows® (Version 1.5, Biotek Instruments, Inc., Winooski, VT).

Statistical Analysis. Data were analyzed using the software program SPSS for Windows©, version 8.0. VEE growth curves (Figure 2) data were analyzed using separate analysis of variances (ANOVAs) at 6, 12, and 24 hours p.i. to determine whether there were treatment group effects. Least Significant Difference (LSD) post-hoc tests were used to compare differences among groups at these time points. Cell viability data (Figure 3) were analyzed using repeated-measures ANOVA with the within-subjects factor of time and between-subjects factor of treatment in order to determine whether treatment groups differed over time. Separate ANOVAs were then conducted at 24, 48, and 72 hours to determine whether groups differed significantly at these time points. LSD post-hoc tests were used to compare differences among groups at these time points. Gene expression data (Figures 5 and 6) were normalized based on controls and analyzed using ANOVAs at each time point with the factor of treatment in order to determine whether groups differed based on treatment. Dunnett's post-hoc tests were used to determine which treatment groups differed significantly from controls. These data also were analyzed with ANOVAs performed on each treatment group with the factor of time in order to determine whether treatment effects differed depending on time point

measured. Dunnett's post-hoc tests were also used to determine which time points within a specific treatment group (*i.e.*, groups measured at 6, 12, 24, and 48 hours) differed from the group measured at 2 hours. Assays to determine NO and TNF-α production (Figure 7 and 8) were analyzed using separate ANOVAs at 6, 12, 24, and 48 hours in order to determine treatment effect. LSD post hoc analyses were then used to determine statistical differences among treatment groups. ANOVAs were two-tailed with $p < 0.05$, whereas post-hoc tests were one-tailed because directionality (*e.g.*, increase in gene expression) had been predicted with $p < 0.05$.

Results

Astrocytes are Targets for VEE Infection. Astrocyte cultures infected with VEE for 18 hours revealed a consistent pattern of morphological changes characteristic of apoptosis. Specifically, nuclei became concentric and fragmented, however no specific immunostaining for VEE could be visualized on the cell surface or in the cytoplasm (Figure 1.B). At 24 hours post-infection (p.i.), positive immunostaining for VEE antigen accompanied by early signs of cytolysis was evident (Figure 1.C), and at 48 hours positive immunostaining was most intense with severe cytopathic effects (CPE) of the monolayer (Figure 1.D). In contrast, mock-infected control wells demonstrated healthy cell populations with defined centrally located nuclei and normal cell morphology throughout the experimental time course (Figure 1.A).

Astrocytes Support VEE Replication. Primary astrocytes were infected with two strains of VEE with different pathogenesis, a virulent strain (V3000) and an attenuated strain (V3010), to determine if astrocytes could support VEE replication and if the *in vivo* phenotype influenced replication in culture. Following VEE entry and removal of non-penetrated virus at one hour p.i., both strains replicated rapidly in astrocytes with a peak of released virus at 24 hours p.i. (Figure 2). However, the attenuated strain V3010 demonstrated slower replication rates as compared to the virulent V3000 and this difference was statistically different at 6 hours p.i. ($*p < 0.05$). These data suggest that replication rates in astrocytes may contribute to the *in vivo* phenotype of VEE.

All extracellular VEE titers remained stable for the initial 48 hours p.i. and then began to decline. This decline in VEE titers coincided with the microscopically observed CPE in VEE-infected astrocytes (e.g., cytoplasmic granulation, cell shrinking and

rounding) including the appearance of floating cellular debris in the culture supernatant. The slow decrease in VEE titers in primary astrocyte culture supernatants (1.2 logs over 48 hours) reflects the relative stability of this virus in non-cell-associated, fluid phase at 37ºC.

Cytopathic Effects of VEE Infection. Although early morphologic changes were evident at 18 hours p.i. (Figure 1.B.) there was not a decline in cell viability until 24 hours p.i. (Figure 3). This appearance of CPE occurs at the same time as peak virus titers measured in the supernatant. While both VEE strains produced CPE in astrocytes, the attenuated strains resulted in delayed cytopathology. This trend of delayed CPE was not statistically significant. Despite similar peak virus titers and cytopathology at 72 hours p.i., differences in kinetics in replication and CPE in astrocytes may correlate with the *in vivo* phenotypes of the different VEE strains.

Induction of Pro-inflammatory Genes in Astrocytes following VEE Infection. Because of kinetic differences in replication rates for VEE and CPE in astrocytes we evaluated kinetics in gene induction for two pro-inflammatory molecules following infection with the molecularly cloned VEE strains V3000 and V3010. IFN-γ/LPS treatment was used as a positive control. Both VEE strains (virulent V3000 and neuro-attenuated V3010) induced iNOS and TNF-α genes in astrocyte cultures in response to infection (Figure 4). Statistical analyses of triplicate samples indicated that IFN-γ/LPS-stimulated astrocytes had statistically significant increases in iNOS gene expression as early as 6 hours p.i. as compared to uninfected controls ($*p < 0.05$), whereas both virus-treated groups were not statistically significant from controls until 12 hours p.i. (Figure 5). These levels of iNOS gene induction peaked at 24 hours p.i. and remained higher

throughout the remainder of the experiment for all three groups as compared to uninfected controls.

Comparisons among groups indicated that the IFN-γ/LPS-stimulated astrocytes upregulated gene expression for TNF-α as early as 2 hours post-stimulation and then declined, whereas the virus-infected astrocytes showed slower upregulation of TNF-α message RNA (Figure 6). This upregulation for TNF-α gene expression was statistically significant for V3000 as early as 6 hours p.i. as compared to uninfected controls ($*p < 0.05$). These levels of TNF-α gene induction remained higher throughout the experiment for both virus-infected groups as compared to uninfected controls. Similar to results previously described by others (Chung and Benveniste, 1990), our experiments demonstrated that a combination of IFN-γ and LPS had a synergistic effect on gene expression in astrocytes when compared to individual stimulatory effects by IFN-γ or LPS (data not shown). Finally, our data demonstrate that astrocyte infection with VEE induced consistent levels of TNF-α gene expression when compared to IFN-γ/LPS and that levels of TNF-α gene expression were highest for the virulent V3000.

Production of NO and TNF-α in Astrocytes in Response to VEE Infection. Nitrite assays of culture supernatants from primary astrocytes infected with VEE or stimulated with IFN-γ/LPS demonstrated that nitric oxide was produced at statistically significant levels ($*p < 0.05$, Figure 7) as compared to uninfected controls. Nitrite levels in identical astrocyte cultures infected with either the virulent or the neuro-attenuated VEE clone remained at base line for the first 24 hours p.i. and were only elevated at low levels (5-6 μM/ml) at 48 hours p.i. These low levels of nitrite in VEE-infected astrocytes were statistically significant as compared to uninfected controls ($*p < 0.05$).

Interestingly, there was no difference in NO production between the virulent and attenuated VEE-infected astrocytes. In contrast, the NO production in the positive control treated astrocyte cultures (IFN-γ/LPS) resulted in elevated nitrite levels as early as 12 hours post-stimulation ($*p < 0.05$) and nitrite levels continuously increased over the experimental time course.

Immunoassays of these culture supernatants demonstrated that astrocytes secreted significant amounts of TNF-α when compared to untreated controls ($*p < 0.05$, Figure 8). Infection of identical astrocyte cultures with virulent V3000 resulted in secreted TNF-α levels of 500 pg/ml, but this peak was not reached until 48 hours p.i. Infection with the neuro-attenuated V3010 resulted in similar TNF-α secretion kinetics, however the levels of TNF-α measured in the supernatants were significantly reduced as compared to V3000 ($**p < 0.05$). Primary astrocyte cultures stimulated with IFN-γ/LPS were used as positive controls and secreted 800 pg/ml of TNF-α as early as six hours post-stimulation ($*p < 0.05$). This peak sharply declined over the next 24 hours and returned to near baseline levels by 48 hours post-stimulation.

Discussion

VEE is capable of infecting primary astrocytes and replicating efficiently, with the consequence of causing substantial cell death. It is well documented that VEE is neuro-virulent (Charles *et al*., 1995, Grieder *et al*., 1995, Jackson *et al*., 1991, Jackson and Rossiter, 1997), however, our *in vitro* experiments, as well as *in vivo* experiments from Jackson & Rossiter (1997), suggest that astrocytes are a potential target for VEE after it has established a productive infection in the CNS. If substantial numbers of astrocytes undergo cell death following VEE infection *in vivo*, these astrocyte-VEE interactions could be a contributing factor to neurodegeneration because essential astrocyte functions are lost. Alternatively, if during the course of VEE infection astrocytes are activated to produce pro-inflammatory cytokines and other diffusible molecular immuno-modulators, this response may be detrimental to neighboring neurons.

Pro-inflammatory responses have been associated with acute neurodegeneration in several different models, including traumatic brain injury and ischemia (Rothwell and Strijbos, 1995) and systemic bacterial infections (Waage *et al*., 1989). Further, inflammatory responses in the CNS have also been linked to specific types of neurological disease, such as autoimmune processes in multiple sclerosis (Benveniste, 1992), AIDS dementia (Adamson *et al*., 1996, Talley *et al*., 1995, Yoshioka *et al*., 1995), or other CNS viral infections (Lieberman *et al*., 1989). Astrocytes and other glial cells during the inflammatory response in the CNS may influence the balance between host protection and neurotoxicity.

Neuro-immune responses of astrocytes to infection by two phenotypically different VEE strains demonstrate that the expression of two pro-inflammatory genes,

iNOS and TNF-α, are induced when compared to uninfected control cultures. These two VEE strains, the molecularly cloned virulent V3000 and the neuro-invasive, but attenuated clone, V3010, differ in their genotype by one single nucleotide (Davis *et al*., 1991). These two molecularly cloned VEE strains with only a single amino acid difference that results in extremely different *in vivo* phenotypes allow us to elucidate cellular responses to infection including cytotoxicity and gene induction in a variety of potential target cell populations. Our finding of slower replication rates in astrocytes for V3010 supports previous findings that slower viral replication rates of V3010 in the periphery may play a role in the attenuated phenotype (Grieder *et al*., 1995).

In previous work, investigating pro-inflammatory gene induction in the periphery in response to VEE infection, we demonstrated that two molecularly cloned VEE strains elicited different cytokine responses when compared to one another. Specifically, an attenuated strain resulted in slower induction kinetics of cytokines, suggesting that a delayed cytokine response may influence the development of host-protection (Grieder *et al*., 1997). In contrast, the present results indicated that two neuro-invasive VEE clones are capable of strongly inducing two important genes in astrocytes, and that the kinetics of gene expression are similar. These similarities in iNOS and TNF-α gene expression following virulent and attenuated VEE infection *in vitro,* yet very different mortality rates following infection with different VEE phenotypes *in vivo* suggest that other factors are involved in the CNS pathogenesis. In the present study, immunoassays for TNF-α demonstrate that additional regulatory mechanisms, such as post-transcriptional events or protein function regulation are important in the final outcome of TNF-α secretion into the supernatant. Further support for post-transcriptional regulation is the observation of low

nitrite levels in VEE-infected astrocyte cultures. Levels of nitrite were much lower than predicted given the quantitation of iNOS gene expression in virus-infected and IFN-γ/LPS-induced control astrocytes. Possible explanations for post-transcriptional regulation of iNOS include the availability of substrates and cofactors essential for the synthesis of iNOS and NO, or other unknown regulatory factors. Such post-transcriptional regulation of iNOS has been demonstrated in other immunocompetent cells (Le Page *et al*., 1996). These observations support the concept that the final outcome of CNS infection with VEE of different phenotypes may not only depend upon differences in virus replication rates and dissemination, but also upon the presence other cell targets such as astrocytes or microglia, and the induction of pro-inflammatory genes at the level of expression or post-transcriptional regulation.

Other investigators have determined pro-inflammatory responses in the brain following infections with closely related viruses. Griffin and colleagues (1994) characterized cytokine expression in the brain in response to the alphavirus *Sindbis virus* (SB) (Wesselingh *et al*., 1994, Tucker *et al*., 1996). Their results demonstrated that pro-inflammatory cytokines play a significant role in the pathogenesis of SB-induced encephalitis and, specifically, that NO may be involved in protecting mice from fatal progression of the disease. *Sindbis virus* has also been utilized to characterize cytokine responses in murine astrocytes *in vitro* (Brodie *et al*., 1997). In contrast to the SB *in vivo* studies, these investigators found that neuro-virulent SB infection in astrocytes induced TNF-α expression, but not iNOS, and the authors concluded that SB infection of astrocytes does not produce enough TNF-α to induce iNOS gene expression. Differences between our findings with VEE and SB infection of astrocytes may include host species

differences, differences between molecularly cloned VEE and biological mutants of SB, and differences in the sensitivity of quantitative methods (*i.e.* semi-quantitative PCR for VEE *versus* Western blots for SB).

Studies investigating *Japanese encephalitis virus* (JEV), a closely related virus in the family Flaviviridae, supports the beneficial role of NO in viral encephalitis (Lin *et al.*, 1997) both *in vivo* and *in vitro*. *In vitro*, inhibition of replication of JEV in IFN-γ-activated murine macrophages was correlated to cellular NO production. *In vivo*, the mortality rate increased as the JEV-infected mice were administered a NOS competitive inhibitor. However, these results with JEV are in contrast to results describing experiments involving another Flavivirus, *Tick-borne encephalitis virus* (TBE) (Kreil and Eibl, 1996). Macrophages from TBE-infected mice spontaneously produced NO *in vitro* and high levels of NO production did not display an inhibitory influence on TBE replication. *In vivo* administration of a competitive inhibitor of NO production to TBE-infected mice significantly increased their mean survival time. These data suggest that NO plays a role in the development of TBE disease and that inhibition of NO formation may be beneficial to the host. These contrasting findings suggest that inducible genes, specifically iNOS, in the CNS have unique responses to similar types of challenges and that there is a low threshold from host protection to toxicity. Further analysis with sensitive quantitative and qualitative methods is necessary to unravel the role of iNOS and NO production, as well as other pro-inflammatory mediators, following viral infections of the CNS.

Primary astrocyte cultures are an important tool in understanding the relationships between glial cells, neurotropic viruses, and cytokines or other diffusible molecules.

These highly homogeneous cultures are useful in isolating responses that may occur during the neurotropic phase of VEE infection *in vivo*. The benefits of using astrocyte cultures as an experimental model for studying CNS viral infections include characterizing replication rates, determining cellular outcomes and neuro-immune responses. Similar models have been used for other neurotropic viruses including *Japanese encephalitis virus* (Suri and Banerjee, 1995, Lin *et al*., 1997), *Sindbis virus* (Brodie *et al*., 1997), *Tick-borne encephalitis virus* (Kreil and Eibl, 1996), and the paramyxovirus *Newcastle disease virus* (Fisher *et al*., 1994, Lieberman *et al*., 1989, Lieberman *et al*., 1990, Rus *et al*., 1992). Moreover, astrocyte cultures have been used to characterize astrocyte responses to specific stimuli aimed at the upregulation of certain molecular factors including NO and TNF-α. Such inducers of NO and TNF-α in astrocytes include IFN-γ, LPS, as well as neurotropic viruses such as *Newcastle disease virus* and *Theiler virus* (Chung and Benveniste, 1990, Feinstein *et al*., 1996, Galea *et al*., 1994, Molina-Holgado *et al*., 1997, Rus *et al*., 1992). Combining primary glial cultures and molecularly cloned VEE with well-characterized phenotypes will help further this progress.

In summary, our results demonstrate that VEE infects astrocytes *in vitro* and that the response of these primary astrocytes to VEE infection includes cytotoxic effects and the induction of pro-inflammatory genes. Because of these findings *in vitro* and the fact that VEE is neuro-invasive, astrocytes are likely targets for infection after VEE has crossed the blood-brain barrier. Even if only a small subset of astrocytes are infected, because of their large numbers and the efficient VEE replication rates, infected astrocytes may play a significant role in terms of increasing intracerebral viral titers and inducing

specific neuro-immune mediators. Astrocyte infection and activation could therefore contribute to the pathogenesis of VEE encephalitis. Understanding the relationship of early neuro-inflammatory responses to VEE infection may help in targeting and manipulation of such responses that are beneficial. Such strategies could have significant application for a variety of neurodegenerative processes that share similar pathways, responses and outcomes.

Acknowledgements

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Table 1. Oligonucleotides Specific for Rat used in PCR and Southern Blots.

Product	Primer/Probe	Sequence (5′ to 3′ direction)	Product Size
GAPDH	sense	CCATGGAGAAGGCTGGGG	195bp
	antisense	CAAAGTTGTCATGGATGACC	
	probe	CTAAGCATGTGGTGGTGCA[1]	
TNF-α	sense	AGAACTCCAGGCGGTGTCTGT	356bp
	antisense	CCTTGTCCCTTGAAGAGAACC	
	probe	ATCAGTTCCATGGCCCAGA	
iNOS	sense	CTGCATGGAACAGTATAAGGCAAAC	229bp
	antisense	GAGACAGTTTCTGGTCGATGTCATGA	
	probe	GGGCTCCAGCATGTACC	

PCR Amplification Specifications

Product	Cycle Number	Annealing temperature	Accession Number	Reference
GAPDH	30	55ºC	M17701	(Tso *et al*., 1985)
TNF-α	28	60ºC	L19123	(Kirisits *et al*., 1994, Shirai et al., 1989)
iNOS	30	63ºC	U03699	(Feinstein et al., 1996, Galea *et al*., 1994)

[1] This was the sequence of the probe used to detect GAPDH sequences following RT-PCR, however it should be noted that this sequence includes two mismatched nucleotides as compared to the original RNA sequence. The correct sequence should read CTAAGCAGTTGGTGGTGCA.

Figure 1. Immunoperoxidase Staining for VEE in Astrocytes

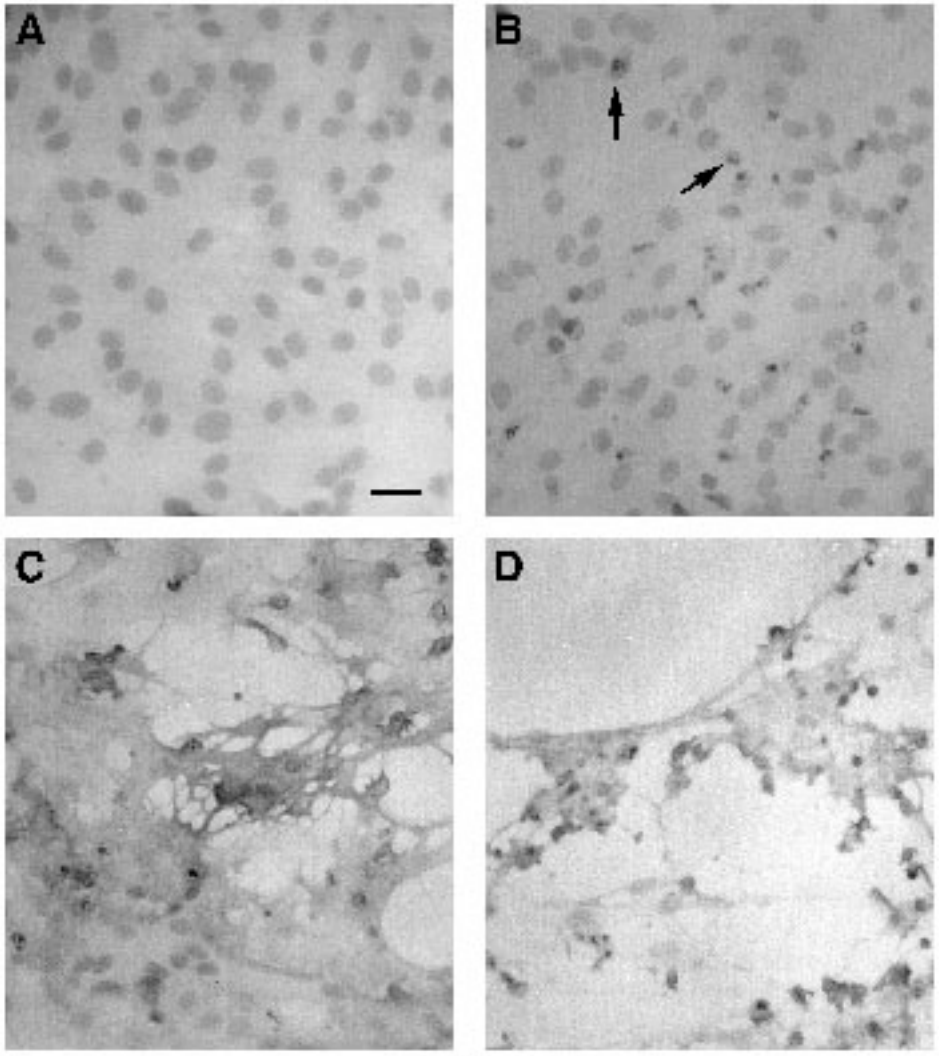

Figure 1. Photomicrograph of immunoperoxidase staining of astrocytes infected with VEE or mock-infected. Mock-infected controls show no staining (Panel A). Early time points post-VEE infection (Panel B, 18 hours) demonstrate morphologic changes in astrocyte cultures including nuclear condensation and fragmentation (arrows), but no positive staining for VEE. Time points 24 and 48 hours (Panel C and D, respectively) reveal positive brown staining for VEE and cytopathic effects to primary astrocyte cultures. Scale bar = 10 μm.

Figure 2. VEE Growth Curves in Astrocytes

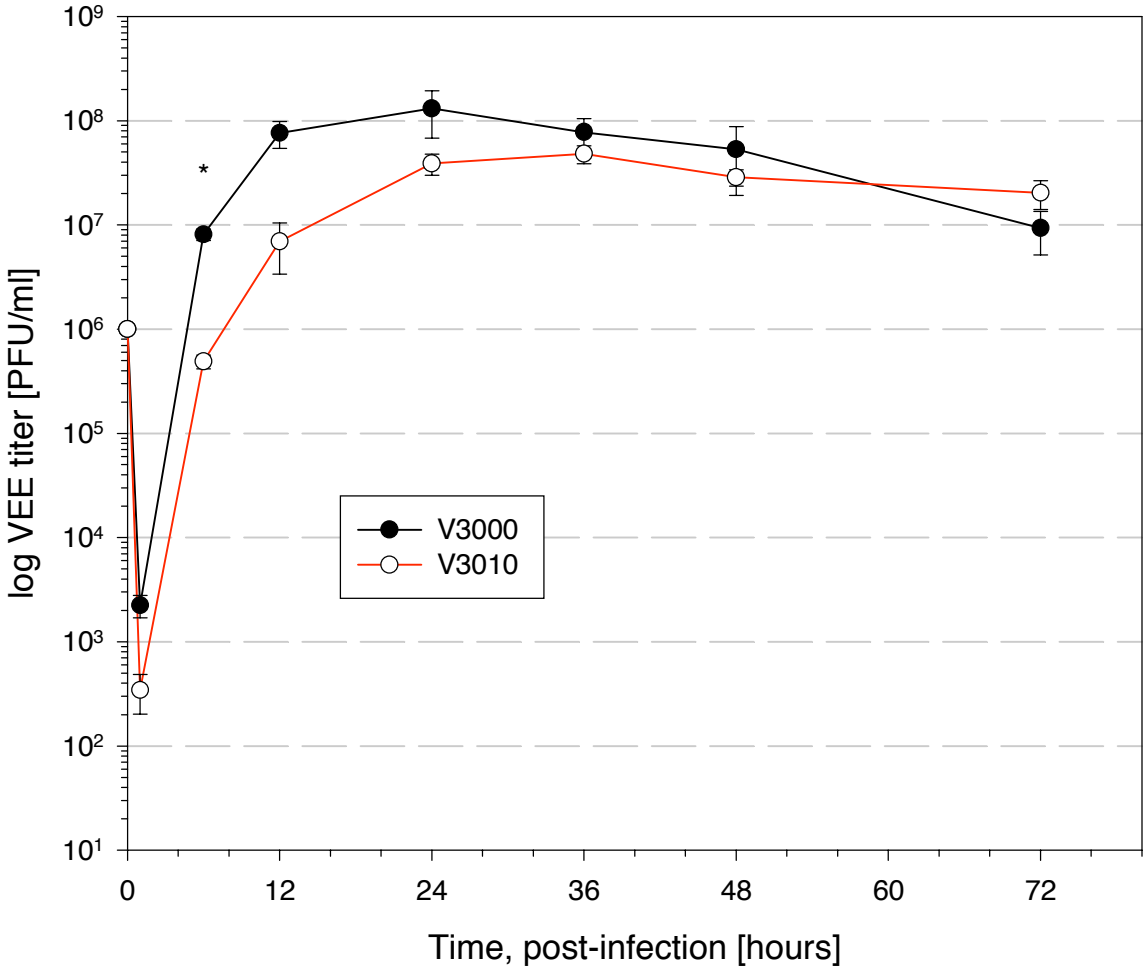

Figure 2. Astrocyte cultures were infected at a multiplicity of infection of 1.0, and VEE titers in the culture supernatant were determined at 1, 6, 12, 24, 36, 48 and 72 hours p.i. Virulent and attenuated VEE strains demonstrate rapid, logarithmic growth within the first 24 hours. Attenuated V3010 demonstrated a statistically significant slower rate of replication at 6 hours p.i. (*$p < 0.05$) as compared to virulent V3000.

Figure 3. Cytopathic Effects of VEE in Astrocytes

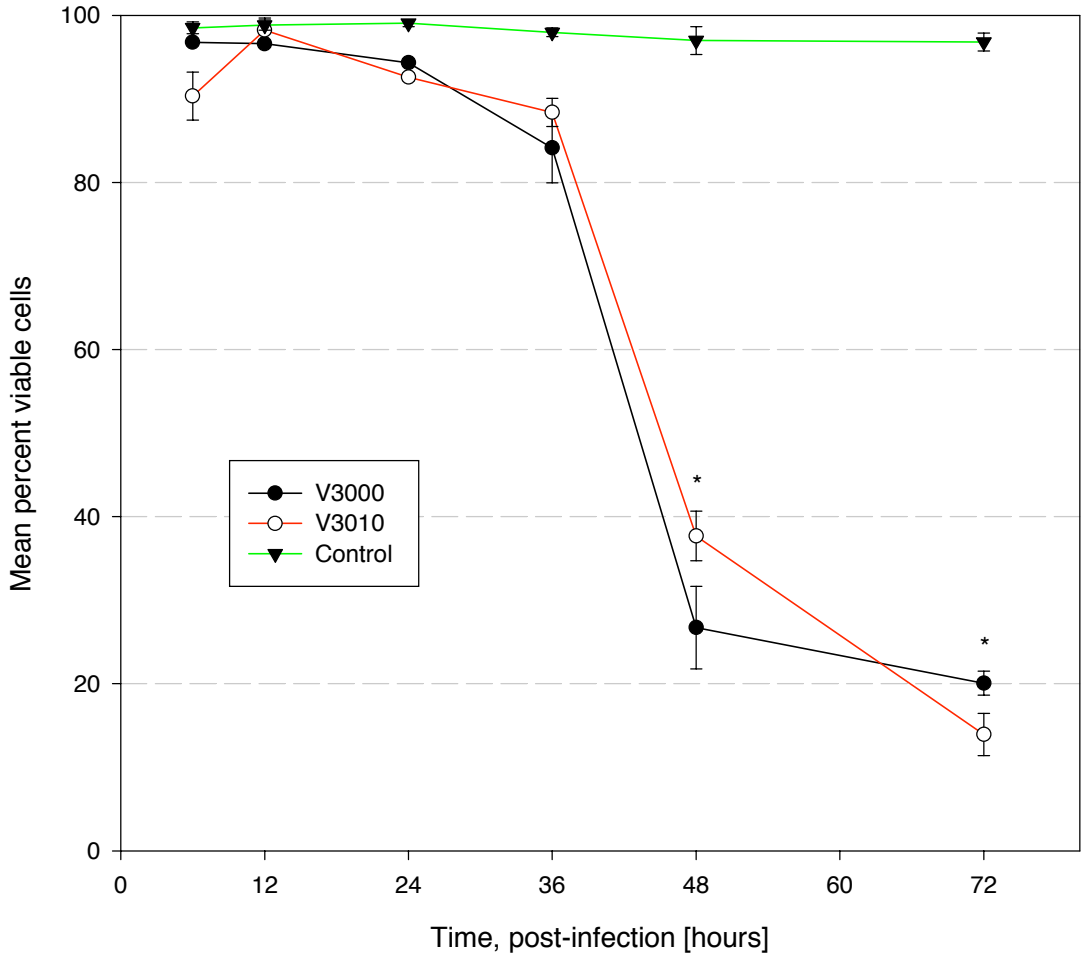

Figure 3. Astrocyte cultures were infected at a multiplicity of infection of 1.0 with virulent and attenuated VEE strains and cell viability was determined by Trypan Blue exclusion test. Mean percents ± SEM of viable cells were determined by three independent samplings along with mock controls for each time point. Both VEE strains were cytopathic for astrocytes (*$p < 0.05$) as compared to uninfected controls.

Figure 4. Southern Blots of iNOS and TNF-α Expression in Astrocytes

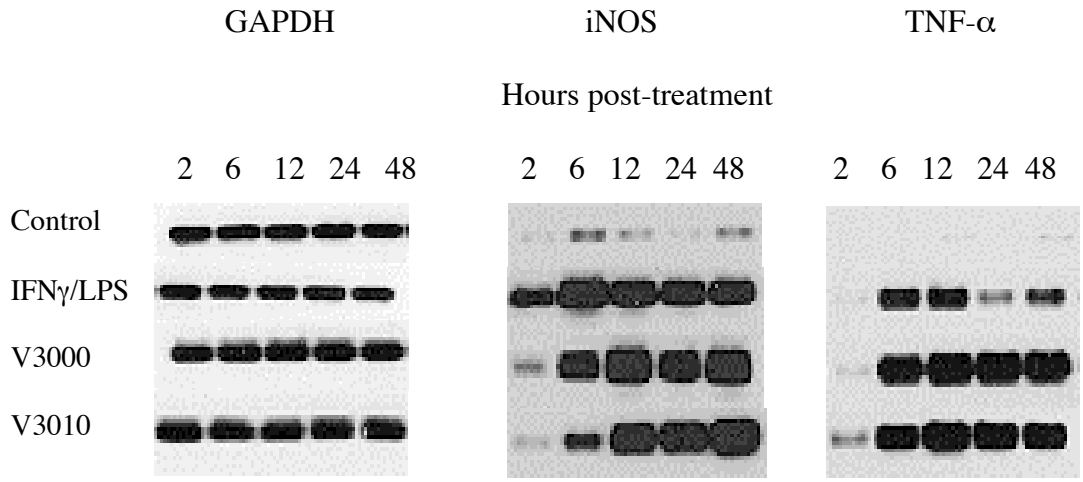

Figure 4. Southern blots for gene expression of glyceraldehyde-3-phosphate dehydrogenase (GAPDH), inducible nitric oxide synthase (iNOS), and tumor necrosis factor alpha (TNF-α). Astrocytes were infected with virulent V3000 or neuro-invasive, attenuated clone V3010 at a multiplicity of infection of 1.0. Both strains of VEE demonstrate gene induction as early as 6 hours p.i. The housekeeping gene GAPDH remains unchanged throughout the experiment.

Figure 5. iNOS Gene Expression in Astrocytes

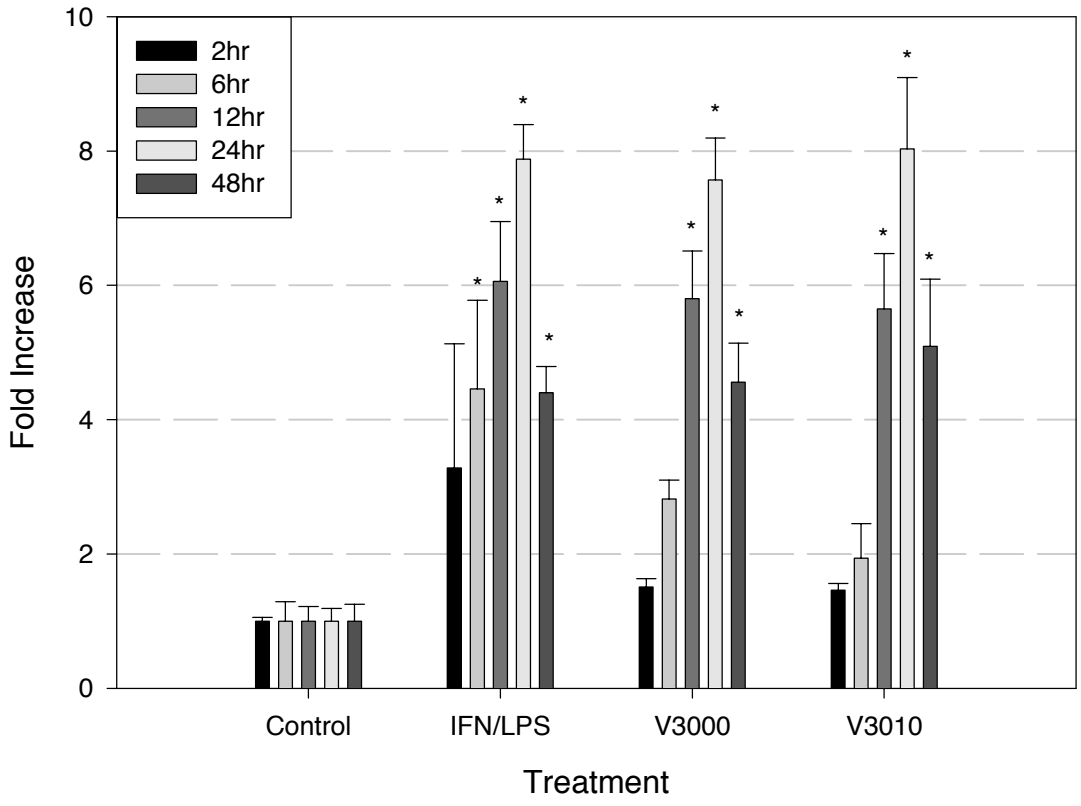

Figure 5. Histogram depicting changes in gene expression for iNOS based on optical density measurements in response to VEE infection. Bars represent fold increase as compared to untreated controls (means ± SEM) of three independent samples. Both virulent and attenuated strains for VEE, as well as IFN-γ/LPS, were capable of significant induction for iNOS (*$p < 0.05$).

Figure 6. TNF-α Gene Expression in Astrocytes

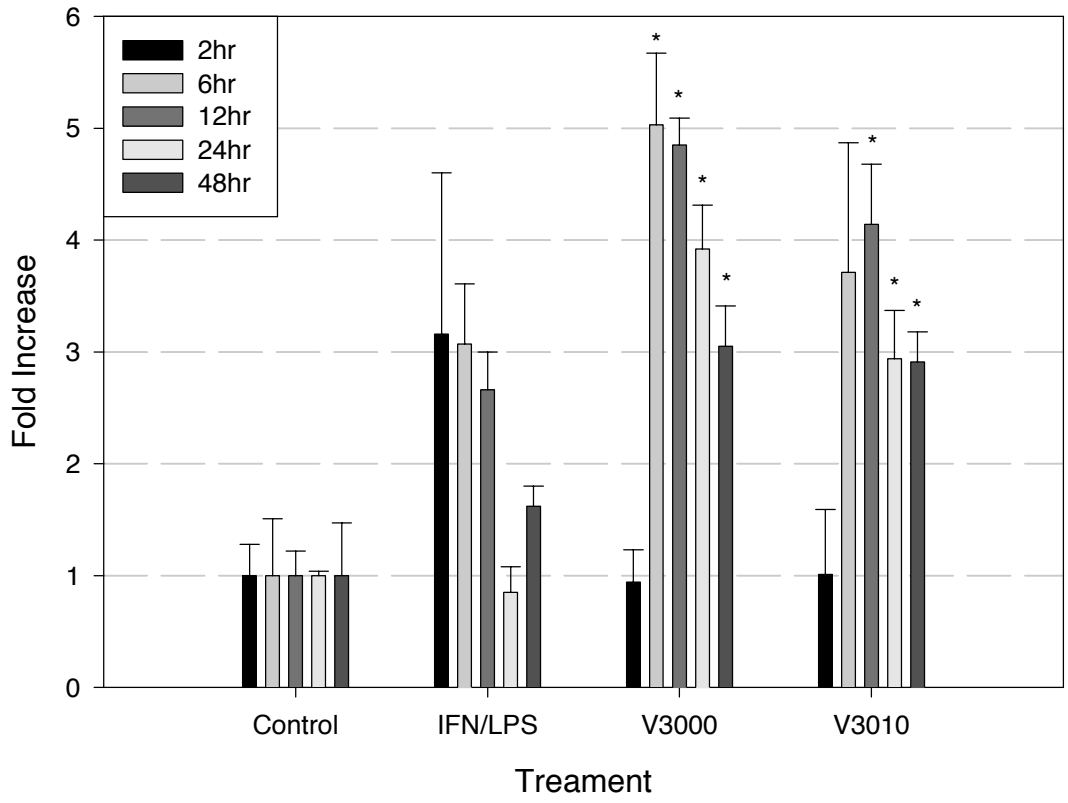

Figure 6. Histogram (mean ± SEM of three independent samples) depicting changes in gene expression for TNF-α based in optical density measurements in response to VEE infection. Virulent and attenuated VEE significantly induced TNF-α gene expression in primary astrocytes (*$p < 0.05$) as compared to uninfected controls. Virulent V3000 upregulated TNF-α gene expression as early as 6 hours p.i., and levels of TNF-α gene expression in both virus genotypes were higher as compared to IFN-γ/LPS stimulated astrocytes.

Figure 7. Nitric Oxide Produced by Astrocytes

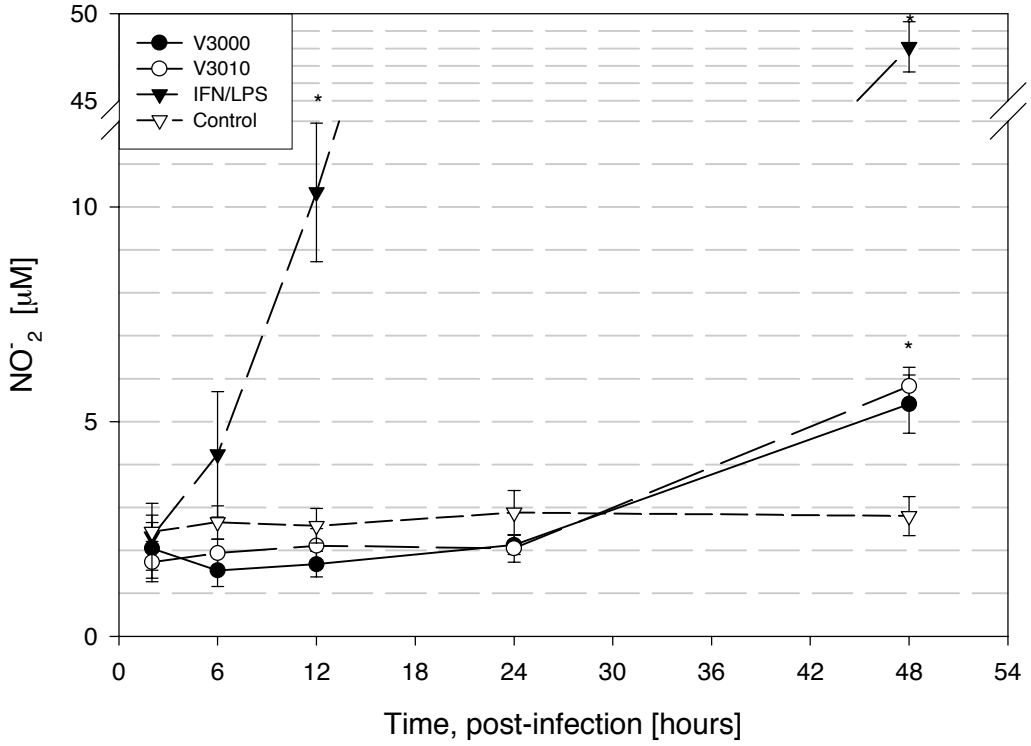

Figure 7. Astrocytes infected with virulent V3000, neuro-attenuated V3010, or stimulated with IFN-γ/LPS showed very different kinetics and levels of nitric oxide production. IFN-γ/LPS stimulated astrocytes produced high levels of nitrite that resulted in final concentrations of 48μM nitrite at 48 hours, whereas VEE-infected astrocytes produced low levels of nitrite (5 μM) that were only detectable at 48 hours p.i (*$p < 0.05$ when compared to uninfected controls).

Figure 8. TNF-α Produced by Astrocytes

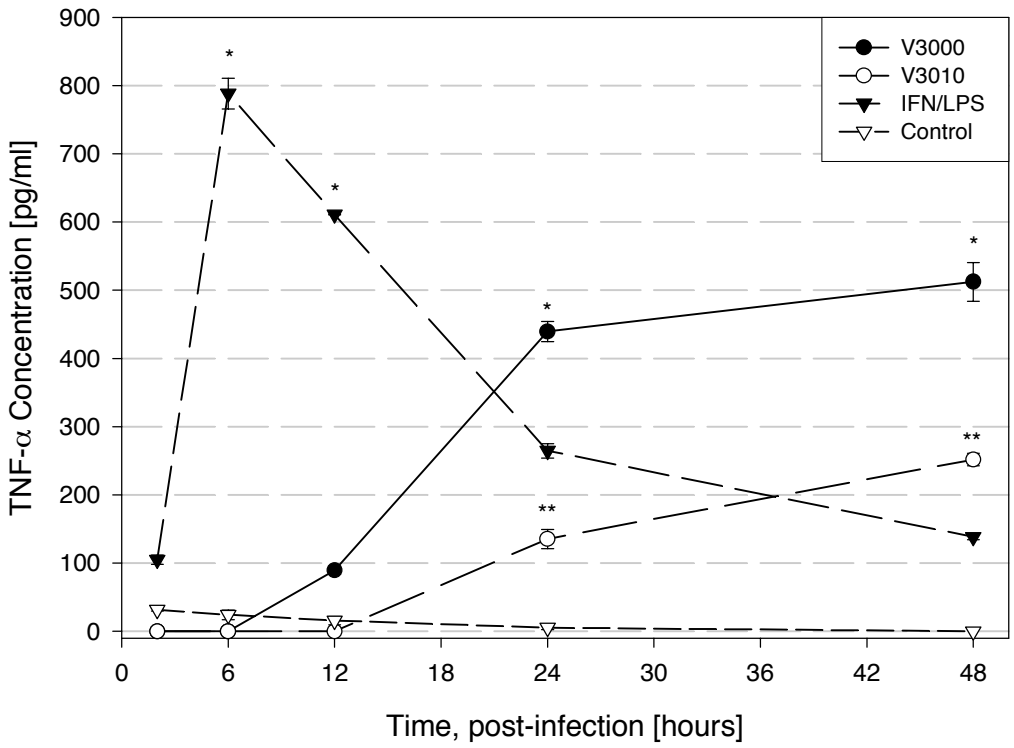

Figure 8. Astrocyte supernatants were analyzed for TNF-α protein levels following infection with virulent V3000 or neuro-attenuated V3010 or stimulated with IFN-γ/LPS. IFN-γ/LPS-stimulated astrocyte production of TNF-α peaked at 6 hours post-stimulation (*$p < 0.05$) and the declined to near baseline levels by 48 hours. VEE-infected astrocyte production of TNF-α peaked at 48 hours p.i. (*$p < 0.05$). Neuro-attenuated V3010 infection of astrocytes resulted in significantly lower levels of TNF-α as compared to virulent V3000 at 24 and 48 hours p.i. (**$p < 0.05$).

CHAPTER 3

Inflammation is a Component of Neurodegeneration in Response to *Venezuelan Equine Encephalitis Virus* Infection in Mice

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Key words: VEE, cytokines, inflammation, neurodegeneration, and apoptosis

Abstract

Infection with the mosquito-transmitted *Venezuelan equine encephalitis virus* (VEE) causes an acute systemic febrile illness followed by meningoencephalitis. In this communication we characterize the cytokine profile induced in the central nervous system (CNS) in response to virulent or attenuated strains of VEE using RNase Protection Assays. Virulent VEE causes an upregulation of multiple pro-inflammatory genes including inducible nitric oxide synthase (iNOS) and tumor necrosis factor alpha (TNF-α). To determine if iNOS and TNF-α contribute to the neuropathogenesis of VEE infection, iNOS and TNF receptor knockout mice were used in VEE mortality studies and exhibited extended survival times. Finally, CNS tissue sections labeled for VEE antigen, and adjacent sections double-labeled for an astrocyte marker and apoptosis, revealed that apoptosis of neurons occurs not only in areas of the brain positive for VEE-antigen, but also in areas of astrogliosis. These findings suggest that the inflammatory response, which is in part mediated by iNOS and TNF-α, may contribute to neurodegeneration following encephalitic virus infection.

Introduction

Arboviruses are a group of emerging pathogens that have been striking communities in the Americas with increasing frequency. Recent examples include outbreaks of *West Nile-like virus* in New York City (MMWR, 1999a) and surrounding communities, and the enzootic nature of *St. Louis encephalitis virus*, *Eastern equine encephalitis virus*, *Western equine encephalitis virus* (MMWR, 1999b) and *Dengue virus* (MMWR, 1999c) with recurring epidemics (Moore *et al*., 1993). Not only are these pathogens capable of producing disease, but they also cause death, particularly among the young, elderly, and immunocompromised populations. To compound the problem, there are no licensed vaccines for these diseases and treatment is limited to supportive measures. The only effective preventive measure is vector control consisting of aerosolization of pesticides, behavior modification including restriction of outdoor activities, and elimination of mosquito breeding grounds such as stagnant water pools.

Venezuelan equine encephalitis virus (VEE) is a well characterized arbovirus for studying the effects of viral encephalitides on the central nervous system (CNS). Advantages of the VEE model system include the availability of molecularly cloned virulent and attenuated strains (Davis *et al*., 1989, Davis *et al*., 1991) and the well established murine animal model (Jackson *et al*., 1991, Davis *et al*., 1994, Grieder *et al*., 1995, Grieder *et al*., 1997). VEE infection causes a biphasic disease with a systemic lymphatic phase and viremia followed by a CNS phase. The end result is virus invasion into the brain parenchyma causing an acute meningoencephalopathy. Although the gross characteristics of VEE neuropathogenesis have been well characterized (Garcia-Tamayo

et al., 1979, de la Monte *et al*., 1985, Jackson *et al*., 1991, Davis *et al*., 1994, Charles *et al*., 1995, Grieder *et al*., 1995, Vogel *et al*., 1996, Jackson and Rossiter, 1997, Steele *et al*., 1998, Grieder and Vogel, 1999) and the peripheral inflammatory response to VEE has been described in detail (de la Monte *et al*., 1985, Grieder *et al*., 1997), little is known about the early immune response in the CNS to VEE infection. Recent studies demonstrate that interferons (IFNs) and interferon regulatory factors (IRFs) are crucial for CNS protection (Grieder and Vogel, 1999, Schoneboom *et al*., 2000b), however this protection is limited in scope.

The central event of VEE infection in the CNS is neuronal degeneration and the mechanism has been hypothesized to be direct infection of neurons. However, pro-inflammatory responses have recently been implicated in neurodegenerative processes. These responses include the upregulation of cytokines and other immunomodulatory genes; the list of such mediators include inducible nitric oxide synthase (iNOS) and its product nitric oxide ($^{\bullet}$NO) (Boje and Arora, 1992, Chao *et al*., 1992, Dawson *et al*., 1993, Merrill *et al*., 1993, Zheng *et al*., 1993, Chao *et al*., 1995, Skaper *et al*., 1995, Chao *et al*., 1996, Kreil and Eibl, 1996), tumor necrosis factor alpha (TNF-α) (Lieberman *et al*., 1989, Chao *et al*., 1995), and the interleukins IL-1α, IL-1β and IL-6 (Chao *et al*., 1995, Rothwell and Strijbos, 1995, Kossmann *et al*., 1996). In this communication we characterized the profile for several inflammatory immunomodulators that are important in containing viral infections, but also have been implicated in neurodegeneration. Further, we demonstrated that astrocytes are activated in response to VEE infection and that this astrogliosis is associated with apoptosis. Characterization of the innate pro-inflammatory immune mechanisms in the CNS following VEE infection is important in

understanding the complexity of the cytokine cascade in the brain in response to viral encephalitides. A better understanding of these cytokines and immunomodulators and their temporal relationships will help target pro-inflammatory responses that have beneficial or detrimental effects for the host with the goal of manipulating those responses for therapeutic benefit.

Materials and Methods

2.1. Virus

Three neuro-invasive, molecularly cloned VEE strains, virulent V3000, attenuated V3010 and V3034 (kindly provided by Dr. Robert Johnson, UNC, Chapel Hill NC), were used for all experiments (Davis *et al*., 1991, Grieder *et al*., 1995). The virulent V3000 clone was derived from a Trinidad donkey strain (Randall and Mills, 1944, Davis *et al*., 1989). The VEE mutant clones were created by site-directed mutagenesis of V3000 targeted at specific sites on one of the two envelope glycoproteins, *E1* and *E2*, which form a heterodimeric spike on the viral envelope surface (Davis *et al*., 1991). The resulting VEE mutants differ from their virulent parent by specifically selected nucleotides, resulting in viruses that differ by only single amino acids (Davis *et al*., 1991, Davis *et al*., 1994, Grieder *et al*., 1995). For V3010, this mutation at glycoprotein *E2* position 76 replaces a glutamic acid in V3000 for lysine and reduces the mortality rate from 100% to 10% in the inbred mouse strain C57BL/6J via a peripheral footpad injection. V3034 has a mutation in *E1* at glycoprotein position 272, in which threonine is substituted for alanine. This mutation results in an attenuated strain with a mortality rate of 20% when injected by the same peripheral route. These mortality rates are slightly higher, but comparable to those reported in the outbred mouse strain CD1 (Grieder *et al*., 1995) and have been confirmed by multiple experiments conducted in our laboratory. Molecularly cloned virus stocks were stored at -80°C and all experiments were conducted in a biosafety level 3 laboratory.

2.2 Primary Astrocyte Cultures

Primary astrocyte cultures were established as previously described (McCarthy and de Vellis, 1980, Schoneboom *et al*., 1999). Astrocyte cultures were characterized by positive immunofluorescent staining for glial fibrillary acidic protein (GFAP; Sigma, St. Louis MO), an intermediate filament which is expressed by astrocytes. Homogeneity was determined by immunostaining identical cultures with a microglia specific cell-surface marker antibody, OX42 (Serotec/Harlan; Indianapolis IN) and an oligodendrocyte specific antibody antiGal-C (Boehringer, Germany). Astrocyte primary cell cultures were determined to be greater than 95 % homogenous by three independent samplings.

2.3 Animals

Six to eight-week-old female C57BL/6J mice (Jackson Laboratory, Bar Harbor ME) were used for the analysis of gross pathology (n = 10 mice per group; cerebral edema), histopathology, double staining for astrocytes and apoptosis (n = 10 mice per group), and RPA analysis (n = 5 mice per group times 3 repeats, for a total of 15 mice per group). Mortality studies used age and sex matched C57BL/6J mice (n = 10 mice per group for background mortality study), C57BL/6-*Nos2*^{tm1Lau} -/- mice (iNOS -/-, n = 5 mice per group), with a targeted mutation of the inducible nitric oxide synthase gene found on chromosome 11 (Jackson Laboratory, Bar Harbor ME), and C57BL/6-*Tnfrsf1a*^{tm1Mak} -/- mice (TNFRp55 -/-, n = 5 mice per group), with a targeted mutation of the TNF receptor p55 gene on chromosome 6 (Jackson Laboratory, Bar Harbor ME).

C57BL/6J mice were used as controls. All mice were lightly anesthetized with methoxyflurane (Pitman Moore Inc., Washington Crossing NJ) and infected with 1 x 10^3 plaque forming units (PFU)/25µl phosphate buffered saline (PBS) with Ca^{2+}/Mg^{2+} and 0.1% donor calf serum via left rear footpad injection with one of the VEE strains or mock infected with PBS. All virus inocula were titrated on BHK-21 cells between passage 53 to 63 (ATTC, Rockville MD) by standard plaque assay (Scherer *et al*., 1971, Grieder and Nguyen, 1996) to confirm virus concentration. Finally, all surviving mice were given a lethal challenge of 1 x 10^4 PFU/100 µl PBS of virulent V3000 via intraperitoneal injection and observed for 14 days post-infection (p.i.) to confirm that these animals had developed a protective immune response against VEE.

2.4 Extraction and Detection of mRNA

Astrocytes were passaged one time, plated into 6-well culture plates at 1 x 10^6 cells per well and incubated at 37°C, 5% CO_2 for two days. Medium was removed and wells were assigned to one of the following groups: 1) uninfected PBS control; 2) treatment with a combination of recombinant rat IFN-γ (100 U/ml; R&D Systems, Minneapolis M) and lipopolysaccharide (LPS; 1 µg/ml equals 200 U/ml; protein free *E.coli* K235 LPS (McIntire *et al*., 1967), kindly provided by Dr. Stefanie Vogel, USUHS, Bethesda MD); and 3) infected with V3000 or V3010 at a multiplicity of infection (MOI) of 1.0 in a total volume of 150 µl and incubated for one hour. Astrocyte culture media was replaced after one hour incubation with assigned treatment. Total cellular RNA was harvested from three independent wells at 2, 6, 12, 24, and 48 hours p.i. using RNAzol™

B (Tel-Test, Inc., Friendswood, TX) as previously described (Chomczynski and Sacchi, 1987, Schoneboom *et al*., 1999).

C57BL/6J mice were infected with virulent V3000, neuro-attenuated V3010, or V3034 at 1 x 10^3 PFU/25 μl PBS or mock-infected with diluent into the left rear footpad. Cerebra were hemisectioned and total cellular RNA was harvested from three mice for each treatment group at 3, 4, 5, 6, and 7 days p.i. Samples were homogenized using a polytron (Kinematica AG, Switzerland) in a final volume of 1 ml of RNAzol™ B. After RNA extraction, the concentration and purity of RNA in each sample was determined spectrophotometrically (Beckman Instruments, Inc., Columbia MD) by assessing the $A_{260/280}$ ratios. Specimens were stored at -80° C until processing by ribonuclease protection assay (RPA) using the Riboquant® System (Pharmigen, San Diego CA).

Four multi-probe templates were used: rNT-1, mCK-2b, mCK-3b, and a custom designed template, to screen for the upregulation of specific genes (Table 1). Briefly, ^{32}P-labeled anti-sense RNA probes were synthesized by *in vitro* transcription and hybridized in excess to 15 μg of target RNA in hybridization buffer at 56° C for 14 - 16 hours, after which free probe and other single-stranded RNA species were digested with RNases. The remaining "protected" RNA probes were extracted, purified, and resolved on a 6% denaturing polyacrylamide gel. The gel was blotted, dried under vacuum at 80°C for one hour, and exposed to Kodak X-OMAT™ AR film (Sigma, St. Louis MO) for variable times ranging from 6 to 24 hours at -80°C to obtain optimal exposure. Autoradiograms were scanned into a digital image and blots quantified by measuring pixel densities as previously described (Schoneboom *et al*., 1999). Briefly, pixel densities were measured using Scion Image software for Windows (Scion Corporation,

Frederick MD) and ratios generated of genes of interest to the constitutively expressed glyceraldehyde-3-phophate dehydrogenase gene (GAPDH). Means (± SEM) were calculated from three independent samples and relative changes in gene expression were then based on comparisons to the mock-infected group. Assay controls included undigested ^{32}P-labeled probes, unprotected RNA treated with RNase, and RNA harvested from resting and LPS-stimulated mouse macrophages.

2.5 Histopathology, Immunostaining, and TUNEL

Animals were infected with V3000, V3034, or mock-infected and CNS tissue including cerebrum, cerebellum, diencephalon, and brainstem were harvested at day 7 p.i. Tissue was fixed in 10% buffered formalin phosphate (Fisher Scientific, Fair Lawn NJ) for 24 hours then sectioned into 3 mm coronal sections and paraffin embedded. Tissue blocks were sectioned onto glass slides (Superfrost® Plus, Fisher Scientific, Pittsburg PA) at a thickness of 5 μm. Hematoxylin and eosin stains were used for histopathology.

For immunoperoxidase staining of VEE antigen, a polyclonal rabbit anti-VEE serum (kindly provided by Drs. George Ludwig and Jonathan Smith, USAMRIID, Ft. Detrick MD) at a 1:400 dilution and an avidin-biotin-conjugated peroxidase kit was used (Vectastain ABC Kit, Burlingame CA) in combination with diaminobenzidine (DAB) and nickel as the chromagen resulting in black staining (Grieder and Nguyen, 1996, Schoneboom *et al*., 1999). Cells were counterstained with either methylene blue or hematoxylin (Sigma, St. Louis MO) to visualize nuclear structures and coverslips applied with Acrytol mounting medium (Surgipath® Medical Industries Inc., Richmond IL).

Controls for non-specific staining included uninfected tissue sections treated with rabbit anti-VEE serum and VEE-infected tissue sections treated with unimmunized rabbit serum incubated at the same dilution as the anti-VEE rabbit serum.

Adjacent tissue sections were double-stained for GFAP, a cytoskeletal intermediate filament protein specific for astrocytes (Bignami and Dahl, 1974, Bock *et al*., 1977, Raff *et al*., 1979, Eng, 1985), and apoptosis using the *in situ* method of detection of DNA fragmentation that labels 3′-OH groups of DNA strand breaks utilizing terminal deoxynucleotidyl transferase (TdT) mediated dUTP nick end labeling (TUNEL) using a NeuroTACS™ II double labeling procedure (Trevigen, Gaithersburg MD). Briefly, slide mounted tissue sections were deparaffinized in xylenes (Sigma, St. Louis MO,) and rehydrated in graded ethanols and 1X PBS. Tissue sections were permeablized in 1X PBS with 0.1% BSA and 0.1% Triton X-100 (Sigma, St. Louis MO) and then non-specific secondary antibody binding sites were blocked with 1X PBS with 0.1% BSA and 1% normal goat serum. Primary rabbit anti-bovine GFAP purified immunoglobulin fraction (Accurate Chemical and Scientific Corporation, Westbury NY) was diluted 1:200 in Neuropore (Trevigen, Gaithersburg MD), placed completely over the tissue sections, and incubated in a humidified chamber at 4°C overnight. Slides were washed three times in 100 mM Tris buffer, pH 7.4, incubated with an affinity purified goat anti-rabbit phosphatase-conjugated secondary antibody (Kirkegaard & Perry Laboratories Inc., Gaithersburg MD) at a 1:20 dilution for one hour at room temperature, and then processed using the HistoMark® RED Substrate System (Kirkegaard & Perry Laboratories Inc., Gaithersburg MD). Slides were washed in deionized water three times to stop the reaction. This first labeling reaction resulted in red labeling of astrocytes.

Controls for nonspecific staining of astrocytes included CNS tissue treated with rabbit serum diluted 1:200 substituted for primary antibody.

To quench endogenous peroxidase activity, tissue sections were immersed in methanol (J.T. Baker, Phillipsburg NJ) with 3% hydrogen peroxide (Sigma, St. Louis MO) final working concentration for five minutes and washed in 1X PBS. Tissue sections were immersed in 1X TdT labeling buffer for 5 minutes and then completely covered in the labeling reaction mix of 1 part TdT dNTPs, 1 part 50X Manganese cation, 1 part TdT enzyme, and 50 parts 1X TdT labeling buffer. Tissue sections were incubated for 60 minutes at 37°C in a humidified chamber. TdT reactions were terminated by immersion of tissue sections in 1X stop buffer for five minutes and then washed two times in 1X PBS. Tissue sections were then covered in a strepavidin-horseradish peroxidase (Strep-HRP) solution at room temperature for ten minutes, washed in 1X PBS two times, and immersed in a DAB-nickel-0.1% hydrogen peroxide solution for 8 minutes. Peroxidase activity was terminated by immersion in deionized water. This second labeling reaction resulted in black labeling of apoptotic cell. Controls for TUNEL staining include: 1) TACS Nuclease-treated control of uninfected CNS tissue to generate DNA breaks for positive labeling with TdT, 2) an unlabeled control of TACS Nuclease-treated uninfected CNS tissue with TdT enzyme omitted to determine the amount of non-specific binding of the Strep-HRP and background DAB labeling, and 3) an experimental negative control (uninfected CNS tissue).

Finally, tissue sections were counterstained by immersion in a 1:10 dilution of Blue Counterstain (Trevigen, Gaithersburg MD) in tap water for two minutes, dipped ten

times in a 0.015% ammonium hydroxide (Allied Chemical, Morristown NJ) in tap water solution, dehydrated through graded alcohols and xylenes and coverslipped in Acrytol.

2.6 Gross Pathology Analysis of Cerebral Edema

Age and sex-matched groups of C57BL/6J mice (n = 10 mice per group) were randomly assigned and weighed to assure homogeneous grouping of animals. Animals were infected via left rear footpad injection with 1 x 10^3 PFU of V3000, V3010, or V3034 or diluent. Mice were sacrificed at 7 days p.i. and brains harvested for weight measurements. Brains were removed to include bilateral olfactory bulbs, cerebra, cerebella, diencephalons, and brainstems, then weighed on a calibrated precision scale (Mettler Instrument Corp, Hightstown NJ).

2.7 Statistical Analysis

Data were analyzed using the software program SPSS for Windows©, version 8.0 (SPSS, Inc., Chicago IL). Mortality studies compared virus-infected groups (V3000 or V3034) between C57BL/6J mice, the background strain, and iNOS -/- or TNFRp55 -/- using t-tests for independent samples. Gene expression and cerebral edema data were analyzed using one way analysis of variance (ANOVA) in order to determine whether groups differed based on treatment. Least Significant Difference (LSD) post-hoc tests were then used to determine which treatment groups differed significantly from controls. All analyses were two-tailed with statistical significance established at $p < 0.05$ *a priori*.

Results

3.1 VEE infection in the CNS induces the upregulation of pro-inflammatory genes

RNase Protection Assays (Table 1) for characterizing the innate immune response in the CNS following VEE infection revealed distinct temporal differences among the different VEE strains (Figure 1). Adult C57BL/6J mice infected with virulent V3000 exhibited an upregulation of several pro-inflammatory genes at the earliest time point collected (3 days p.i.), and this upregulation was maintained throughout the entire experimental time course. In contrast, age and sex-matched mice infected with the neuro-attenuated VEE mutants, V3010 or V3034, demonstrated a distinctly different cytokine profile. Specifically, mice infected with V3034 did not demonstrate an upregulation of pro-inflammatory genes until day 5 or 6 p.i., while mice infected with V3010 had no detectable mRNA cytokine response in the CNS following VEE infection for the entire observation period. These different cytokine profiles do not correlate with the establishment of VEE infection in the CNS. All three phenotypes, virulent V3000, neuro-attenuated V3010 and V3034, are found in the CNS by day 3 p.i., and throughout the experimental time course (data not shown; Grieder *et al*., 1995).

In addition to the temporal differences in gene upregulation among mice infected with different VEE strains, there were also consistent, quantitative differences in their responses (Figure 2). Virulent V3000-infected mice demonstrated a significant difference in the upregulation of several important pro-inflammatory genes, including TNF-α, IL-6, IFN-γ, IFN-β, iNOS, IL-1α/β, and IL-12 as compared to uninfected controls ($p < 0.05$), as well as, from attenuated VEE-infected mice, with the exception of

TNF-α, IL-6 and IFN-β. Both V3000 and V3034 induced TNF-α, IL-6, and IFN-β at significant levels as compared to controls ($p < 0.05$).

3.2 Prolonged survival of iNOS and TNFR knockout mice when infected with VEE

Preliminary mortality studies using the molecularly cloned, neuro-invasive VEE strains and the peripheral footpad inoculation route demonstrated a mortality rate of 100% in C57BL/6J mice infected with virulent V3000 and a 10% and 20% mortality for V3010 and V3034, respectively (Table 2a), consistent with previous reports using biological VEE with inbred and outbred strains of mice (Jackson *et al*., 1991, Grieder *et al*., 1997, Grieder and Vogel, 1999, Grieder *et al*., 1995).

To determine if $^{\bullet}$NO and TNF-α play an important role in the inflammatory response to VEE infection and the eventual outcome of the disease, we compared mortality rates and average survival times (AST) in mice with targeted mutations in either iNOS or TNF receptor p55 genes *versus* the background strain C57BL/6J following infection with either virulent V3000 or attenuated V3034 (Table 2b and 2c). This study revealed that there were significant differences in the AST when comparing iNOS -/- and C57BL/6 mice infected with virulent VEE, even though mortality rates were unchanged. The AST in iNOS -/- mice infected with virulent V3000 was 2.6 days longer as compared to C57BL/6J mice ($p < 0.05$). iNOS -/- mice infected with the attenuated V3034 appeared to have reduced mortality, however, the number of mice tested does not allow for statistical analysis. When comparing TNFR -/- and C57BL/6J mice following infection with either virulent V3000 or attenuated V3034, the TNFR -/-

mice showed a trend in prolonged survival time for both virus strains. Taken together, these results suggest that $^{\bullet}NO$ and TNF-α contribute to the pathogenesis of VEE encephalopathy as pro-inflammatory early immune responses in the CNS.

3.3 VEE infection alters neurotrophic support in astrocytes

To elucidate if gene expression for neurotrophic support is altered in response to VEE infection, RNA from primary rat astrocytes infected with VEE was analyzed. Results from this analysis demonstrated that rat astrocytes respond to VEE infection *in vitro* with the upregulation of two neurotrophins (Figure 3); brain-derived neurotrophic factor and glial cell line-derived neurotrophic factor (BDNF and GDNF, respectively) gene expression were significantly increased in the attenuated V3010-infected astrocytes when compared to uninfected controls ($p < 0.05$). These findings suggest that neurotrophic support is altered in glial cells in response to VEE infection. The role of regulating neurotrophic support in response to VEE infection in neuronal survival *in vivo* is unknown.

3.4 VEE infection in the CNS causes marked encephalitis and astrogliosis

Hematoxylin and eosin stained coronal brain sections of mice harvested 7 days p.i. with virulent VEE showed classic signs of viral encephalopathy including meningoencephalitis and vasculitis with lymphocytic infiltrates into the neural parenchyma. Several areas also showed necrosis and vacuoles of parenchyma, however no liquefaction was observed (data not shown).

Positive immunocytochemical staining for VEE antigen demonstrated areas associated with inflammation, necrosis, and vacuolization (Figure 4A and B). VEE antigen was detected as cytosolic staining almost exclusively in neurons as characterized by morphology. Such areas of VEE-positive staining appeared to be randomly distributed throughout the brain and included every area of the CNS in V3000-infected mice including the cortex, basal ganglia, diencephalon, cerebellum, and brainstem. Furthermore, there were areas of the brain that showed acute signs of inflammation without positive VEE antigen staining (Figure 4E and F). This finding suggests that the inflammatory response to VEE infection is not restricted to cells and surrounding areas where VEE infection or active replication is occurring. This observation of widespread inflammation beyond the boundaries of VEE-antigen detection may function as a mechanism to contain viral spread within the CNS. Finally, VEE-infected brains revealed a remarkable astrogliosis characterized by the proliferation or hypertrophy of astrocytes with increased intensity of staining for GFAP (Figure 4C, D and F); this astrogliosis was significant as compared to uninfected controls. In adjacent tissue sections, astrogliosis was demonstrated in all areas of VEE infection, and in areas of acute inflammation where no VEE antigen could be detected.

3.5 Mice infected with VEE have cerebral edema

Adult C57BL/6J mice infected with VEE showed evidence of marked cerebral edema as measured by brain weight on day 7 p.i. Specifically, brain weights of mice infected with virulent V3000 or attenuated V3034 measured 457.6 ± 4.9 and 464.3 ± 4.2 mg, respectively, and these differences were statistically significant ($p < 0.05$). In

contrast, cerebral weights of age and sex-matched control mice and mice infected with V3010 were measured to be 438.4 ± 6.4 mg and 442.0 ± 3.3 mg, respectively. Interestingly, although the brain weights of V3000 and V3034 were similar, these mice appeared to be very different behaviorally at day 7 p.i. All V3000-infected mice showed marked signs of acute encephalitis to include lethargy, recumbency and rigidity, spastic paralysis of the extremities, as well as hematuria. In contrast, V3034-infected mice continued to exhibit normal social behaviors including cage exploration, feeding, grooming, and huddling. Three of ten V3034-infected mice appeared slow and one mouse had hindlimb paralysis. None of the ten V3034-infected mice had gross evidence of hematuria. However, the V3034-infected mice did have dramatic weight loss over the 7 day time course from a pre-infective mean weight of 17.3 g to 14.4 g seven days p.i.

3.6 VEE infection in the CNS results in upregulation of genes important in apoptosis resulting in apoptosis of neurons but not glial cells

Analysis of genes important in apoptosis by RPA demonstrated that several genes were upregulated following VEE infection (Figures 1 and 5). Mice infected with virulent V3000 demonstrated an upregulation of ligands important in initiating the apoptosis signaling cascade including TNF-α, Fas ligand (Fas L), and TNF-related apoptosis inducing ligand (TRAIL), and also members of the family of receptors responsible for transduction of these signals, including p55, p75 and Fas antigen. Similar to the analysis of pro-inflammatory genes, the genes involved in apoptosis signal transduction were significantly upregulated ($p < 0.05$) following V3000 infection as compared to control.

Additionally, their upregulation was earlier (3 days p.i.) and stronger following virulent V3000 as compared to the attenuated VEE strains.

Apoptotic cells with neuronal morphology were identified using TUNEL staining in virulent V3000-infected CNS sections (Figure 4D and F). TUNEL-staining cells were more numerous in V3000-infected CNS sections as compared to uninfected controls and areas of TUNEL-staining were often associated with areas positive for VEE antigen. However, TUNEL staining was also demonstrated in areas of the brain with astrogliosis and signs of inflammation that did not stain positive for VEE antigen (Figure 4E and F). Finally, very few astrocytes showed positive TUNEL staining, indicating that these cells participate in the pathogenesis of the CNS disease, rather than succumb to it.

Discussion

This communication provides new insight into the mechanisms of neurodegeneration following VEE infection. We describe the pro-inflammatory and pro-apoptotic profile of multiple genes upregulated in viral encephalitis, and that these responses may have deleterious consequences for neurons. Mortality studies using iNOS and TNF-α knockout mice suggest that these two immunomodulators may play significant roles in VEE neuropathogenesis. We further demonstrate that astrocytes are a component of this inflammatory response and are activated in areas of the brain where there is apoptosis. Finally, we demonstrate that VEE infection in the CNS causes significant cerebral edema, and that the amount of edema is dependent on the phenotype of VEE infection. These findings suggest that pro-inflammatory and pro-apoptotic gene upregulation, altered neurotrophic support, astrocytosis, and cerebral edema are components of VEE neuropathogenesis.

Our results expand on earlier work demonstrating the important role of IFNs, both type I and II, and IRFs in the immune response of the CNS following VEE infection (Grieder *et al*., 1997, Grieder and Vogel, 1999, Schoneboom *et al*., 2000b). Mice with a disruption in the IFN-α/β receptor gene (IFNAR -/-) were much more susceptible to both virulent and attenuated VEE strains as demonstrated by rapid dissemination of the virus to the brain and very short survival times, whereas mice deficient in IRF-1 and IRF-2 were more susceptible to virulent VEE infection as compared to control mice.

In the present study, we demonstrate that IFN-β and IFN-γ are significantly upregulated in the CNS following VEE infection and that the quantitative and qualitative

response is associated with the VEE strain. Mice infected with virulent V3000 had a significant upregulation of IFN-β detected on day 3 p.i., followed by IFN-γ on day 5 p.i. This temporal pattern of cytokine induction of type I interferon, followed by type II interferon (*i.e.* IFN-γ), fits the multistage induction of immediate-early IFNs (Marie *et al.*, 1998) and the kinetics of IFN-γ previously described in the periphery (Grieder *et al.*, 1997) in response to virulent or attenuated VEE strains. In addition, attenuated strains of VEE showed a delayed (V3034) or absent (V3010) cytokine response, even though virus of all three neuroinvasive phenotypes were present in the CNS by day 3 p.i. (data not shown) (Grieder *et al.*, 1995). This altered temporal and quantitative difference in cytokine expression may play a role in the lower mortality rates associated with the two attenuated VEE strains.

The temporal pattern of the pro-inflammatory response is also linked to the role of type I interferons and the induction of iNOS. Previous studies have shown that IRF-1 and IRF-2 participate in the regulation of IFN-α/β and iNOS (Salkowski *et al.*, 1996, Schoneboom *et al.*, 2000b). In this study, we have demonstrated that IFN-β is upregulated early in the CNS on day 3 p.i. in mice infected with V3000, followed by the upregulation of iNOS on day 4 p.i. This temporal relationship is also demonstrated for the attenuated strain V3034, although the timing of initiation of the response is delayed.

Furthermore, we show that there are multiple pro-inflammatory responses of the CNS to VEE infection and that this complex system of cytokines is interdependent. Specifically, the mortality studies using genetically altered mice with mutations in either their TNFp55 receptor or iNOS genes exhibited increased survival times in both of these knockout strains. These results suggest that these two immunomodulators may also play

a role in the neuropathogenesis of VEE infection. In comparison, results from previous studies utilizing TNFR -/- mice to elucidate the role of TNF in the inflammatory response have been mixed. Homozygous TNFR -/- mice were resistant to lethal challenges of LPS, yet were susceptible to *Listeria monocytogenes* infection (Pfeffer *et al*., 1993). TNF-α also appears to be important in other types of infections. In studies investigating TNF-α in the protective immune response against *Mycobacterium tuberculosis* (MTB), mice lacking a TNF-α response, either by antibody neutralization or by TNFRp55 disruption, were more susceptible to MTB infection and demonstrated significant necrosis in the lung (Flynn *et al*., 1995). This increased susceptibility was also demonstrated for the intracellular pathogen, *Yersinia enterocolitica*, where TNFRp55-/- mice displayed impaired macrophage function. Infected mice showed decreased $^{\bullet}$NO production and impaired oxidative burst activity, which further demonstrated that TNF-α signaling through TNFRp55 controls the severity of Yersinia-induced arthritis. These findings implicate TNF-mediated macrophage microbicidal activity as a central event in anti-Yersinia defense (Zhao *et al*., 1999). Therefore, of major interest is the role of the inflammatory response in disease development. This is illustrated by the development of TNF-α inhibitors for the treatment of rheumatoid arthritis (Moreland, 1999). Our results using an encephalitic viral model of acute inflammation in the CNS suggest that TNF-α plays two important roles: 1) a protective early immune response as a defense to viral infection and, 2) over-expression contributing to the neuropathogenesis.

The role of iNOS in the innate immune response to viral infections is also mixed. Previous studies investigating the role of iNOS in viral infection have shown that iNOS and $^{\bullet}$NO production have beneficial effects for the host against *Coxsackie B3 virus*

(Lowenstein *et al*., 1996), *herpes simplex virus* (MacLean *et al*., 1998), or infection with virulent *Ectromelia virus* (Karupiah *et al*., 1998a); in contrast iNOS and •NO appear to contribute to the pathogenesis of pneumonitis in *influenza A virus* (Karupiah *et al*., 1998b).

Our data investigating specific innate immune responses, parallel those of other investigators exploring the role of the inflammatory response to VEE infection in severe combined immunodeficient (SCID) mice (Charles *et al*., 2000). We both demonstrate that survival times can be increased following virulent VEE infection, however overall mortality is unchanged. These results suggest that the robust inflammatory process may be detrimental to the host, but necessary in an attempt to contain viral spread. Taken together our studies comparing cytokine responses among VEE strains and mortality studies using wild-type (C57BL/6J mice) and knockout mice (TNFR -/- and iNOS -/-) suggest that the pro-inflammatory induction of multiple cytokines and immunomodulators, such as iNOS, may participate in the neuropathogenesis of VEE infection.

Our results demonstrating a significant pro-inflammatory response with multiple genes upregulated in the CNS when infected with VEE are supported by other studies investigating cytokine profiles of neurotropic virus infections. Specifically, studies characterizing cytokine responses in the CNS of 4-week-old BALB/cJ mice with virulent and attenuated strains of a related alphavirus, *Sindbis virus* (SB), demonstrated a robust pro-inflammatory response characterized by the upregulation of IL-1β, IL-6, TNF-α, and IFN-γ (Wesselingh *et al*., 1994). In contrast to our findings of differential cytokine upregulation comparing virulent and attenuated VEE strains, SB infection in the CNS

induced a similar cytokine response, regardless of the viral phenotype and a correlation between cytokine response and severity of disease could not be demonstrated. These differences could be attributed to virus strain, strain and age of mouse model, and experimental approaches to quantitate mRNA expression.

Studies with *Borna disease virus* found upregulation of TNF-α, IL-1α/β, and IL-6, and further demonstrated that this inflammatory response originates from resident cells in the CNS, such astrocytes and microglia (Sauder and de la Torre, 1999). Cytokine profiles comparing virulent and attenuated strains of mouse hepatitis virus JHM also found an early upregulation of TNF-α in virulent JMH-infected mice, transient increases in mRNA for IL-12, IL-1α/β, IL-6, and iNOS, and differential responses between virus phenotypes (Parra *et al*., 1997). Furthermore, chronic infection with JHM in mice resulted in an upregulation of a similar panel of pro-inflammatory mediators (TNF-α, IL-1β, IL-6, and iNOS) in astrocytes in the spinal cord. Consistent with our findings, astrogliosis was not limited to areas in the CNS positive for viral antigen, but also found in areas without JHM infection (Sun *et al*., 1995).

Apoptosis has also been described as a prominent phenomenon that occurs in the CNS in response to viral infections. One hypothesis for the occurrence of apoptosis is that programmed cell death is an altruistic defense strategy of cells to prevent viral replication (Vaux and Hacker, 1995). Alternatively, virus-induced apoptosis may result from indirect mechanisms. Such mechanisms may include the inability of infected cells to effectively maintain gene transcription and protein synthesis resulting in the activation of the death pathway (Tolskaya *et al*., 1995). Specifically, SB has been investigated for its role in the induction of apoptosis in neurons (Griffin *et al*., 1994, Lewis *et al*., 1996).

The conclusion from these studies was that apoptosis occurs in neurons due to direct infection by SB, and reflects neurovirulence as apoptosis correlated with mortality. This event is significant in the CNS because neurons are post-mitotic and have very limited ability to regenerate.

Other studies investigating the role of apoptosis in the CNS following VEE infection describe apoptosis in areas of the brain where VEE antigen was detected (Jackson and Rossiter, 1997), suggesting that apoptosis occurs as a direct consequence of VEE infection in neurons. Our investigation of CNS tissues infected with VEE using double-labeling for astrocytosis and apoptosis, and staining for VEE antigen in adjacent tissue sections, has demonstrated that apoptosis occurs not only in areas of the brain where VEE antigen could be detected, but also in areas of acute astrogliosis and inflammation where no VEE antigen could be demonstrated. This finding suggests that other mechanisms, in addition to direct infection of neurons, are involved in VEE-induced apoptosis in the CNS. The lack of TUNEL-staining astrocytes in the brain of VEE-infected mice and the proliferation of astrocytes associated in areas of marked apoptosis also suggest that glial cells participate in, or are activated by, this type of cell death.

Astrocytes, which are in close proximity to neurons, and in vast numbers, outnumbering neurons by a ratio of 10:1, have the potential to produce innate immune products in response to VEE infection that may be toxic to neighboring neurons. Astrocytes have been shown to alter their function from one of maintenance and neurotrophic support to an immune function (Lieberman *et al*., 1989, Chung and Benveniste, 1990, Benveniste, 1992, Brodie *et al*., 1997, Mendez E, 1997, Schoneboom

et al., 1999), with the production of cytokines and reactive nitrogen species, such as $^{\bullet}$NO. A robust pro-inflammatory response in close proximity to neuronal populations may be neurotoxic and has been hypothesized in many injury models of the CNS (Boje and Arora, 1992, Dawson *et al*., 1993, Chao and Hu, 1994, Chao *et al*., 1995, Pulliam *et al*., 1995, Skaper *et al*., 1995, Yoshioka *et al*., 1995, Kong *et al*., 1996, Vernadakis, 1996, Merrill and Benveniste, 1996). Our findings support the concept that the inflammatory response of the CNS, specifically astrocyte activation and proliferation, may contribute to the neuropathogenesis of VEE infection along with the direct infection of neurons.

Table 1. RPA Templates[a]

rNT-1	mCK-2b	mCK-3b	Custom template
βNGF	IL-12p35	TNF-β	iNOS
BDNF	IL-12p40	LTβ	Fas antigen
GDNF	IL-10	TNF-α	TNFRp75
CNTF	IL-1α	IL-6	IL-1α
NT-3	IL-1β	IFN-γ	IL-1β
NT-4	IL-1Ra	IFN-β	IL-1Ra
L32	IL-18	TGFβ1	Fas2L (TRAIL)
GAPDH	MIF	TGFβ2	TNFRp55
	IL-6	TGFβ3	L32
	IFN-γ	MIF	GAPDH
	L32	L32	
	GAPDH	GAPDH	

[a]RNase Protection Assay Templates used to analyze brain or astrocyte samples for inducible genes. Four templates were used which included a neurotrophic factor template (rNT-1), two templates for cytokines (mCK-2b and mCK-3b), and a custom template for iNOS and genes important in apoptosis. Each template includes two constitutively expressed genes, L32 and GAPDH, which are used as internal controls.

Table 2. Mortality Studies of Mice Strains infected with VEE

C57BL/6J Mortality Study[a]

Virus Strain	*n*	AST ± SEM (Days)	Mortality (%)
V3000	10	8.1 ± 0.3	100
V3010	10	10	10
V3034	10	8.5 ± 1.0	20

iNOS -/- Mortality Study[b]

Mouse Strain	Virus Strain	*n*	AST ±SEM (Days)	Mortality (%)
C57BL/6	V3000	5	7.6 ± 0.4	100
iNOS -/-	V3000	5	10.2 ± 0.5*	100
C57BL/6	V3034	5	9	20
iNOS -/-	V3034	5	N/A	0

TNFR -/- Mortality Study[c]

Mouse Strain	Virus Strain	*n*	AST ±SEM (Days)	Mortality (%)
C57BL/6	V3000	5	9.6 ± 0.4	100
TNFR -/-	V3000	5	10.1 ± 0.4	100
C57BL/6	V3034	5	8.0	20
TNFR -/-	V3034	5	10.0	20

[a]Six to eight-week-old female C57BL/6J mice were used to determine average survival times (AST ± SEM), as measured in days, and mortality rates (percentage of deaths) when infected with 1 x 10^3 PFU of virulent (V3000) or one of two attenuated VEE strains (V3034 or V3010) by left rear footpad injection. [b]Comparison of C57BL/6J to iNOS knockout mice (iNOS -/-) with either virulent V3000 or attenuated V3034, and [c]TNFR knockout mice (TNFR -/-). All of the mortality study experiments were conducted independently.

Figure 1. Six to eight-week-old female C57BL/6J were infected with 1 x 10^3 PFU of virulent V3000 or one of two attenuated VEE strains, V3034 or V3010, by left rear footpad injection or mock-infected (n = 15 mice per group). Brain hemisections were harvested at 3, 4, 5, 6, and 7 days p.i., and total RNA was analyzed by RNase Protection Assay (one of three independent assays shown for 1 of 3 different mice per treatment group). Controls include: undigested probes serving as size markers, unprotected RNA treated with RNase (RNase digest controls), RNA harvested from resting and LPS-stimulated macrophages. V3000-infected mice showed upregulation of multiple genes at 3 days p.i., whereas V3034-infected mice did not demonstrate upregulation until day 5 or 6 p.i. There is no upregulation of pro-inflammatory or pro-apoptotic genes with V3010 throughout the experimental time course as compared to uninfected control mice.

Figure 1.

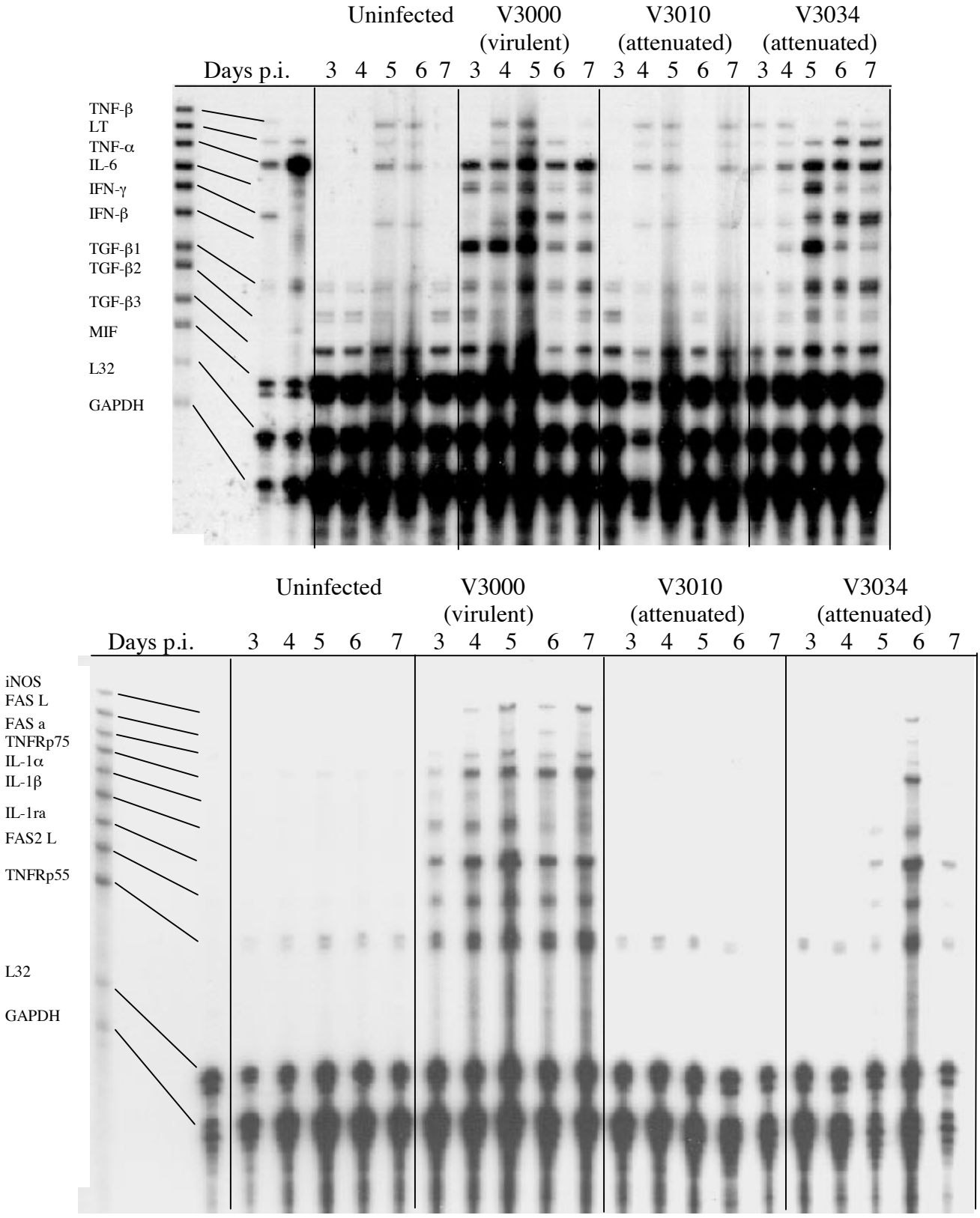
Uninfected
V3000 (virulent)
V3010 (attenuated)
V3034 (attenuated)
Days p.i. 3 4 5 6 7 3 4 5 6 7 3 4 5 6 7 3 4 5 6 7
TNF-β
LT
TNF-α
IL-6
IFN-γ
IFN-β
TGF-β1
TGF-β2
TGF-β3
MIF
L32
GAPDH
Uninfected
V3000 (virulent)
V3010 (attenuated)
V3034 (attenuated)
Days p.i. 3 4 5 6 7 3 4 5 6 7 3 4 5 6 7 3 4 5 6 7
iNOS
FAS L
FAS a
TNFRp75
IL-1α
IL-1β
IL-1ra
FAS2 L
TNFRp55
L32
GAPDH

Figure 2.

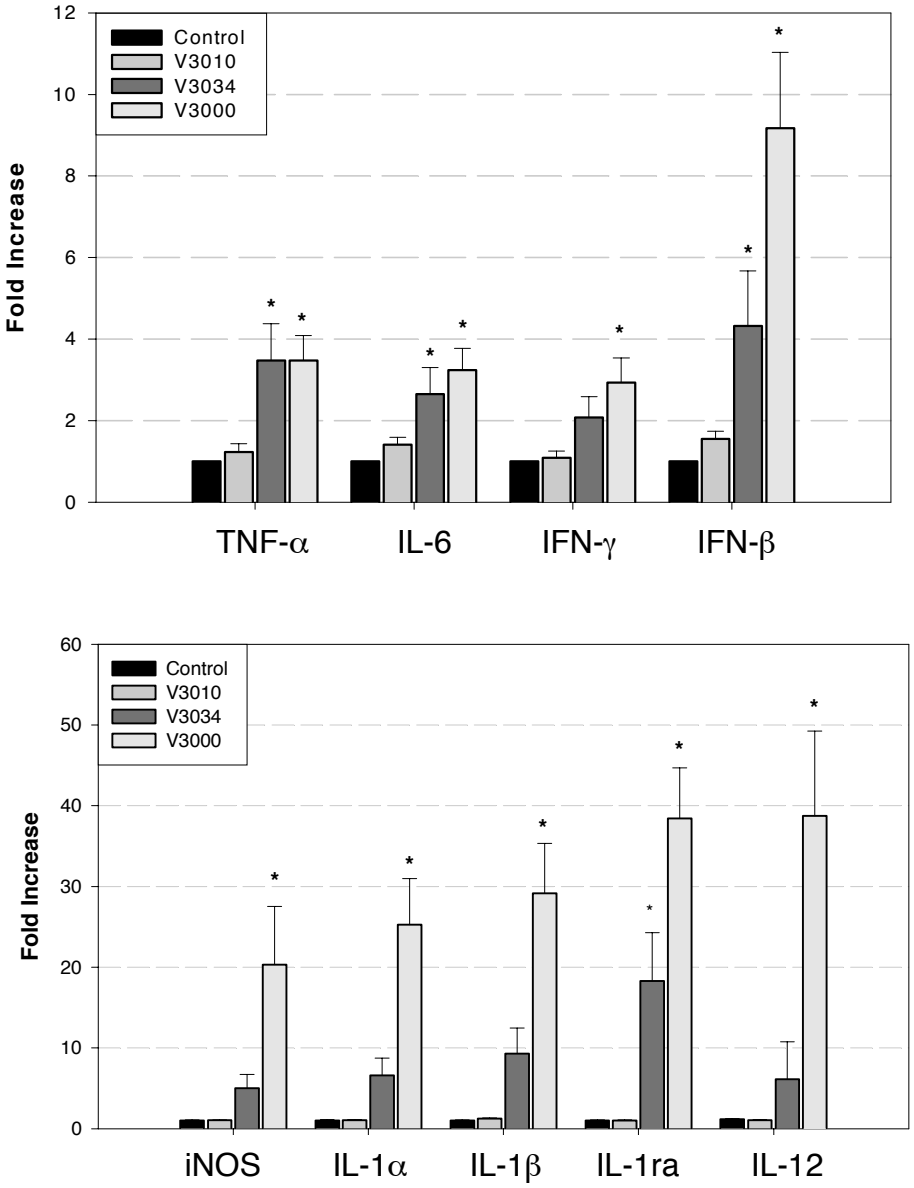

Figure 2. Six to eight-week-old female C57BL/6J mice were infected with 1 x 10^3 PFU with one of the VEE strains (attenuated V3010 or V3034, or virulent V3000) via left rear footpad injection and brains harvested daily on days 3-7 p.i. Gene expression was quantitated as a total for the entire experimental time course by measuring pixel densities of autoradiograms from three independent assays (n = 15 mice per group) and determining ratios of inducible genes over a constitutively expressed gene (GAPDH) and expressed as relative fold increase (mean ± SEM) as compared to uninfected controls. Pro-inflammatory genes were significantly upregulated for all genes tested for V3000, and for TNF-α, IL-6, IFN-β, and IL-1ra for attenuated V3034 (*$p < 0.05$).

Figure 3.

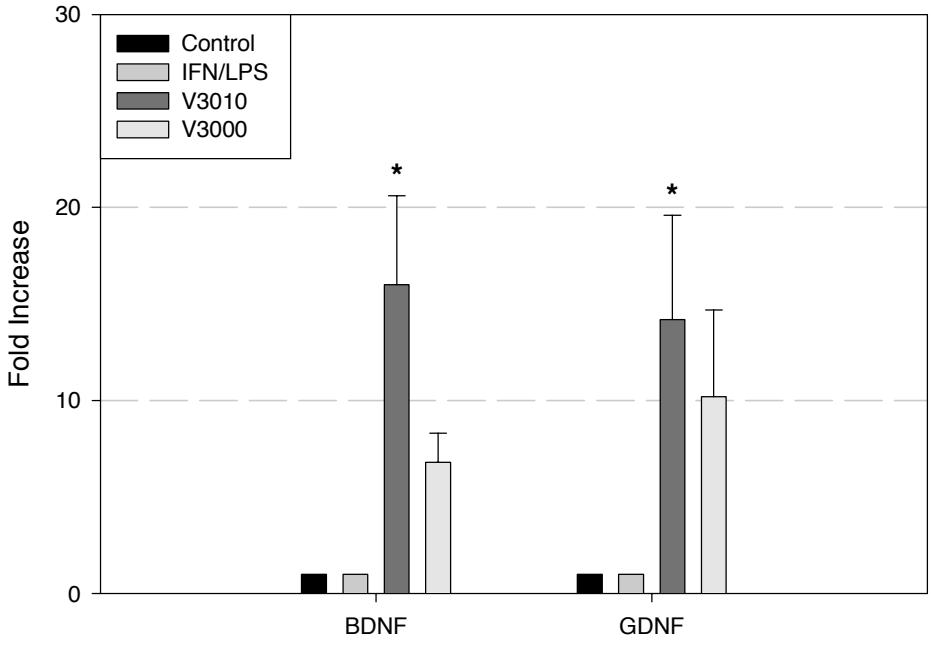

Figure 3. Primary rat astrocytes were infected with V3000 or V3010 at a multiplicity of infection 1.0. Total cellular RNA from triplicate samples was collected at 2, 6, 12, 24, and 48 hours p.i., and processed by RNase Protection Assay using the neurotrophic factor template rNT-1 (see Table 1). Pixel densities of autoradiograms were expressed as a total of relative fold increase for the entire experimental time course. Both virulent V3000 and attenuated V3010 upregulated gene expression of two neurotrophic factors, brain-derived neurotrophic factor (BDNF) and glial cell line-derived neurotrophic factor (GDNF); these were statistically significant for attenuated V3010 ($*p < 0.05$).

Figure 4. Composite photomicrograph of coronal brain sections from an adult C57BL/6J mouse 7 days post-VEE infection. Immunoperoxidase staining resulting in VEE-antigen positive cells (arrow, black stain) with methylene blue counterstain (A, bar = 500 μm). High magnification of the VEE-positive region (B). The cell morphology of VEE staining appears to be neurons (arrows, black staining in the cytoplasm, bar = 50 μm). Adjacent tissue section (C) double-labeled for astrocytes (red stain) and apoptosis (black stain, bar = 500 μm). Hematoxylin was used as a counterstain. High magnification (D) shows significant astrogliosis (red stain, arrowheads) and apoptosis (black stain, arrows; bar = 100 μm). High power field of lateral cortex in the same tissue section shown in A; no positive staining for VEE antigen is detected (E, bar = 100 μm). Adjacent tissue section double-labeled for astrocytes (red stain, arrowheads) and apoptosis (black stain, arrows) demonstrating significant astrogliosis and apoptosis in this area of the brain where VEE-antigen could not be detected (F, bar = 100 μm).

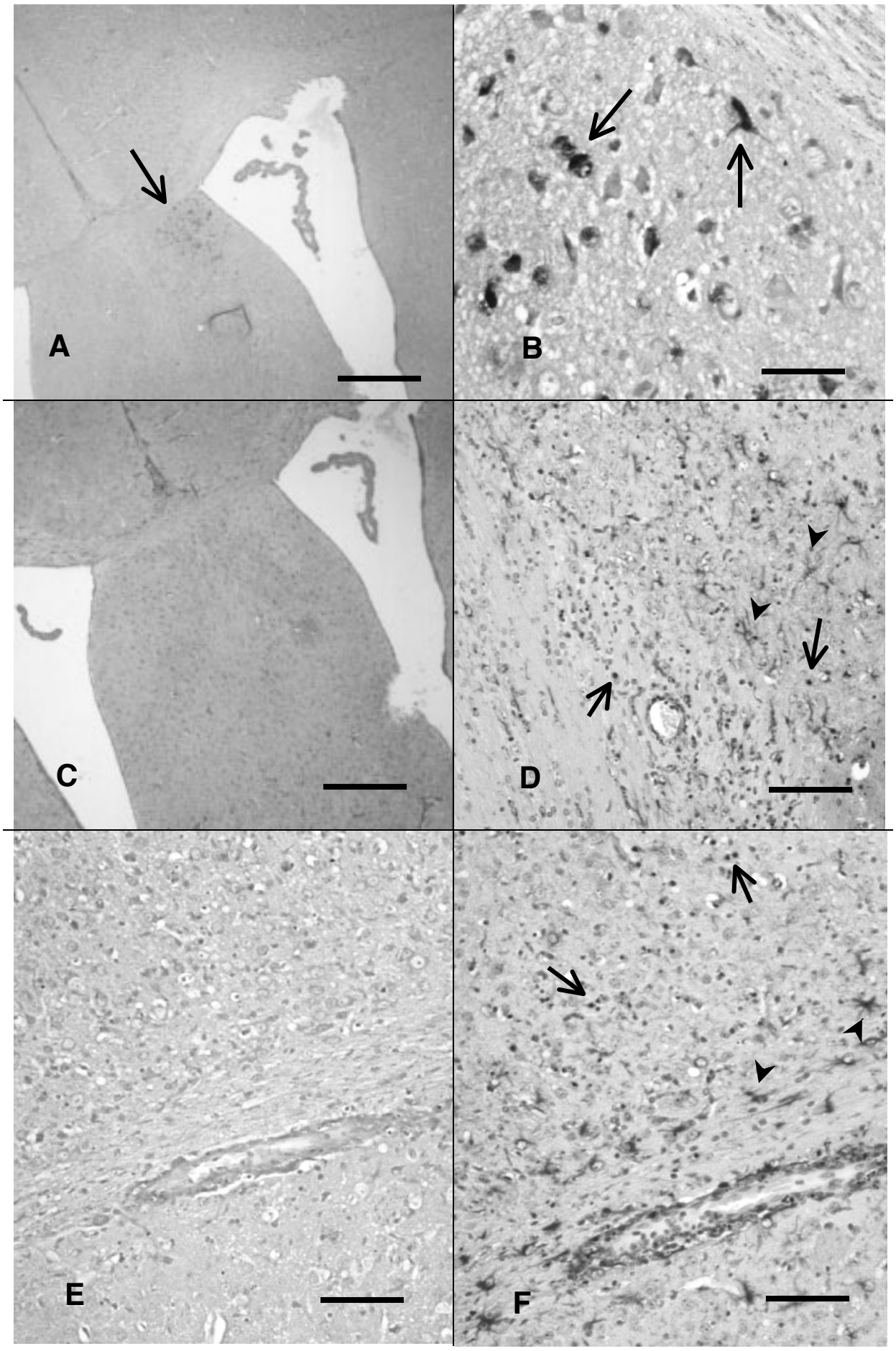
A
B
C
D
E
F

Figure 5.

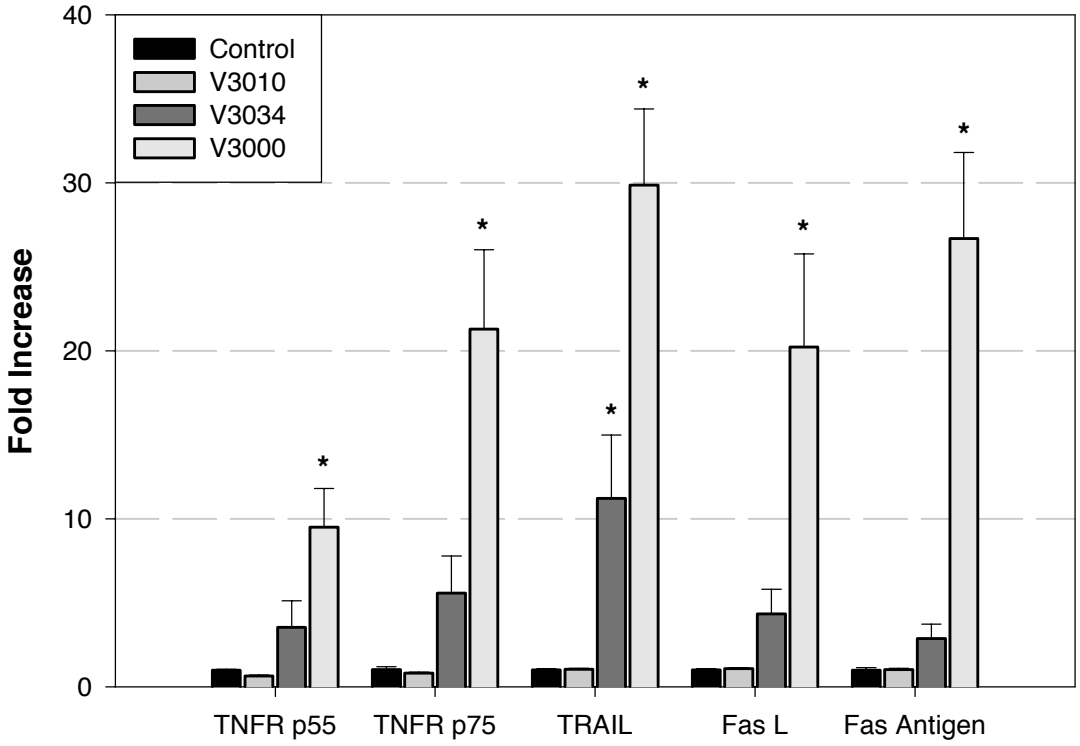

Figure 5. Age and sex-matched C57BL/6J mice were infected with 1 x 10^3 PFU with one of the VEE strains (attenuated V3010 or V3034, or virulent V3000), or mock-infected (control) via left rear footpad injection and brains harvested daily on days 3 - 7 p.i. Gene expression was quantitated as a total for the entire experimental time course by measuring pixel densities of autoradiograms from three independent assays (n = 15 mice per group) and determining ratios of inducible genes over a constitutively expressed gene (GAPDH). Values expressed as relative fold increase (mean ± SEM) as compared to untreated controls. Pro-apoptotic genes were significantly upregulated for all genes tested for V3000 (*$p < 0.05$).

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CHAPTER 4

Additional Results of Neuro-immune Responses to VEE Infection

Further analysis of primary astrocyte cultures infected with VEE demonstrated that astrocytes respond to VEE infection with the upregulation of IL-6. This was demonstrated by RT-PCR for IL-6 mRNA (Figure 1) and by ELISA to measure protein secreted in the culture supernatant (Figure 3). Quantitative analysis of gene expression revealed that astrocytes infected with virulent V3000 or attenuated V3010 upregulated gene expression for IL-6 as early as 6 hours p.i., and maintained this upregulation throughout the experimental time course (Figure 2). The levels of fold increase for IL-6 gene expression approached four-fold 12 hours p.i. for both V3000 and V3010 ($*p < 0.05$). Further, ELISA data demonstrated that IL-6 production was significantly different between the two VEE phenotypes (Figure 3). Astrocytes infected with virulent V3000 responded by producing IL-6 that peaked at 200 pg/ml at 24 hours p.i., and this production was statistically significant as compared to mock-infected controls ($*p < 0.05$). In contrast, astrocytes infected with attenuated V3010 produced significantly lower amounts of IL-6 (peak amount measured of 140 pg/ml at 24 hours p.i.) as compared to virulent V3000 ($**p < 0.05$). This V3010-induced upregulation of IL-6 was, however, still significantly different from uninfected controls ($*p < 0.05$).

In reviewing the literature, the role of IL-6 production in the CNS is mixed. Investigations into functional relationships of IL-6 and nerve growth factor (NGF) utilizing cultures of murine primary astrocytes demonstrated that IL-6 induced NGF production (Kossman *et al*., 1996). The conclusions from these investigations were that IL-6 production in response to brain injury may contribute to the upregulation of

neurotrophic factors by astrocytes suggesting a neuroprotective role for IL-6. In contrast, IL-6 has been associated with several neurological disorders including viral meningitis, suggesting a prominent role for IL-6 in the neuropathogenesis of CNS pathology (Gruol and Nelson, 1997). Further, a transgenic murine model that overexpresses IL-6 in the CNS develops severe neuronal and vascular pathologies, and behavioral abnormalities (Campbell *et al*., 1993). Histopathologically, transgenic mice that overexpress IL-6 in the CNS demonstrate astrogliosis, neurodegeneration, and disruption of the BBB with the development of cerebral edema (Brett *et al*., 1996). These findings of both IL-6 mediated neuroprotection and neurotoxicity indicate that this cytokine is a component of the complex orchestration of cytokines and immuno-modulators that determine the delicate balance between repair and injury in the CNS. My findings of IL-6 production by astrocytes in response to VEE infection and the upregulation of certain neurotrophic factors (BDNF and GDNF) *in vitro* suggests that there are immune responses in the CNS that attempt to balance inflammation. Interestingly, RPA analysis of brain hemisections from mice infected with virulent VEE did not demonstrate the upregulation of any neurotrophic factor analyzed (Figure 4). This finding of unaltered gene expression of neurotrophic factors by RPA in response to VEE infection may indicate that neurotrophic support is dysregulated, further supporting the notion that IL-6 is involved in the neuropathogenesis of VEE infection. Another possible explanation of unaltered neurotrophic support in the CNS in response to VEE infection may be due to the sensitivity of the assay. Other techniques, such as RT-PCR, may be able to demonstrate subtle differences in gene expression because of amplification of mRNA. Alternatively, *in situ* hybridization for specific neurotrophic factors in VEE-infected CNS tissue may be

able to localize areas where neurotrophic support is upregulated. Future experiments should also determine regulation of neurotrophic factors in response to infection over an experimental time course with the attenuated VEE mutants to investigate if neurotrophins play a role in host-protection.

Virus titers in the CNS were also measured following left rear-footpad injection with virulent V3000, attenuated V3010, or V3034 to test if the presence of virus in the CNS is linked to the temporal pattern of the upregulation of inflammatory mediators (Figure 5). This analysis demonstrated that regardless of the VEE phenotype, all strains of VEE have established an infection in the CNS on day 3 p.i. Further, these results corroborate earlier findings characterizing virus penetration and replication in the CNS in outbred strains of mice (Grieder *et al*., 1995). The analysis of VEE titers in the CNS also demonstrated that VEE infection in the brain persisted throughout the experimental time course for all three neuro-invasive VEE strains. These data, along with the RPA analyses, suggest that the neuro-immune response to VEE infection is uniquely dependent on the specific VEE strain and does not correlate with virus penetration and replication in the CNS. Specifically, considering the RPA results (Chapter 3, Figure 1), pro-inflammatory and pro-apoptotic genes were upregulated following V3000 infection at the earliest time point analyzed (3 days p.i.) and this response was maintained throughout the experimental time course. In contrast, these responses were delayed or non-detectable following infection with V3034 or V3010, respectively, despite the fact that both of these VEE phenotypes have established CNS infections by 3 days p.i., and that these two attenuated strains were not cleared from the CNS by the end of the experimental time course (day 7 p.i.).

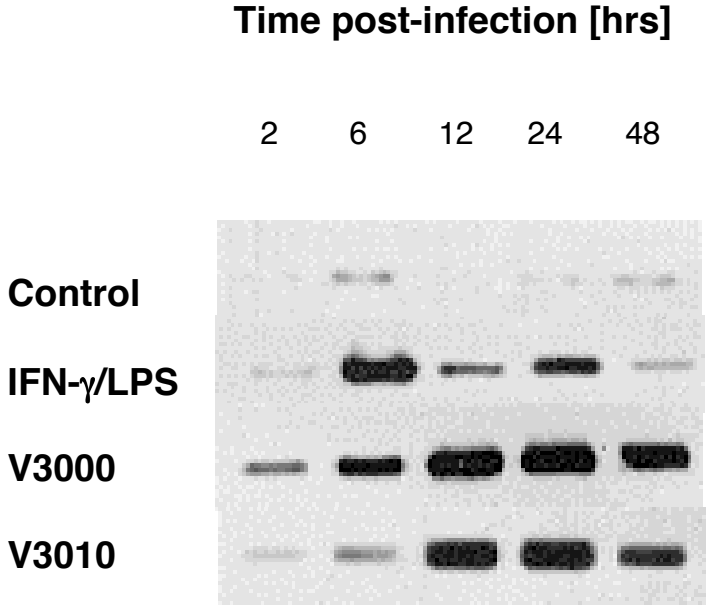
Analysis of cerebral edema also demonstrated that VEE infection in the CNS induced swelling of the brain (Figure 6). Specifically, both V3000 and V3034 caused significant cerebral edema as compared to mock-infected controls ($*p < 0.05$), however there was no difference demonstrated between these two phenotypes (virulent V3000 and attenuated V3034). This was surprising considering the differences in inflammatory response determined by RPA and the clinical features of encephalitis between these two VEE phenotypes. All V3000-infected mice showed marked signs of acute encephalitis to include lethargy, recumbency and rigidity, spastic paralysis of the extremities, as well as hematuria. In contrast, V3034-infected mice continued to exhibit normal social behaviors including cage exploration, feeding, grooming, and huddling. Three of ten V3034-infected mice appeared slow and one mouse had hindlimb paralysis. None of the ten V3034-infected mice had gross evidence of hematuria. Considering these very different clinical features, total brain weight analysis may not be the best test to evaluate cerebral edema. Other methods to determine cerebral edema that may be a more accurate measure include percentage of brain weight to total body weight, ratios of wet to dry brain weights, or an experimental time course of brain weight measurements. This time course analysis would be able to determine if there are differences in the development and duration of cerebral edema that occur between VEE phenotypes. Such a time course might demonstrate that both peak brain weight and the duration of increased brain weight are different between V3000 and V3034 infected mice.

Finally, quantitative analysis of apoptosis and astrogliosis in the CNS of mice infected with either virulent V3000 or attenuated V3034 demonstrated that these measures of neuropathogenesis correlated with the VEE phenotype (Figure 7).

Specifically, CNS tissue harvested at day 7 p.i. from both V3000- and V3034-infected mice, and double-labeled for GFAP and TUNEL, had higher numbers of positive staining cells for both astrocytes and apoptosis as compared to uninfected controls ($*p < 0.05$). Furthermore, there were significant differences when comparing the number of GFAP and TUNEL-labeled cells following virulent V3000 and attenuated V3034 infection. CNS tissue from V3000-infected mice had a higher number of positive staining cells by both measures as compared to attenuated V3034 ($**p < 0.05$).

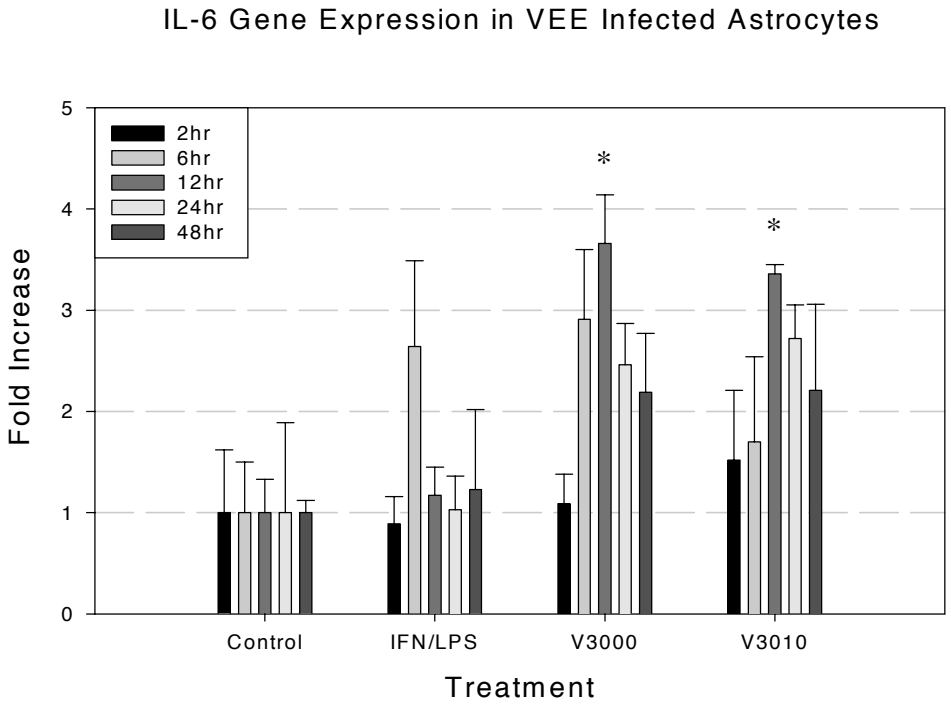
In summary, the findings of IL-6 production by astrocytes *in vitro* in response to VEE infection, along with the *in vivo* investigations of CNS viral burden, neurotrophic support, cerebral edema, and measures of astrogliosis and apoptosis parallel the findings described in the previous chapters. Taken together they suggest that the inflammatory response contributes to the pathogenesis of VEE encephalitis and that there is a pro-inflammatory threshold between neuroprotection and neurotoxicity.

Figure 1



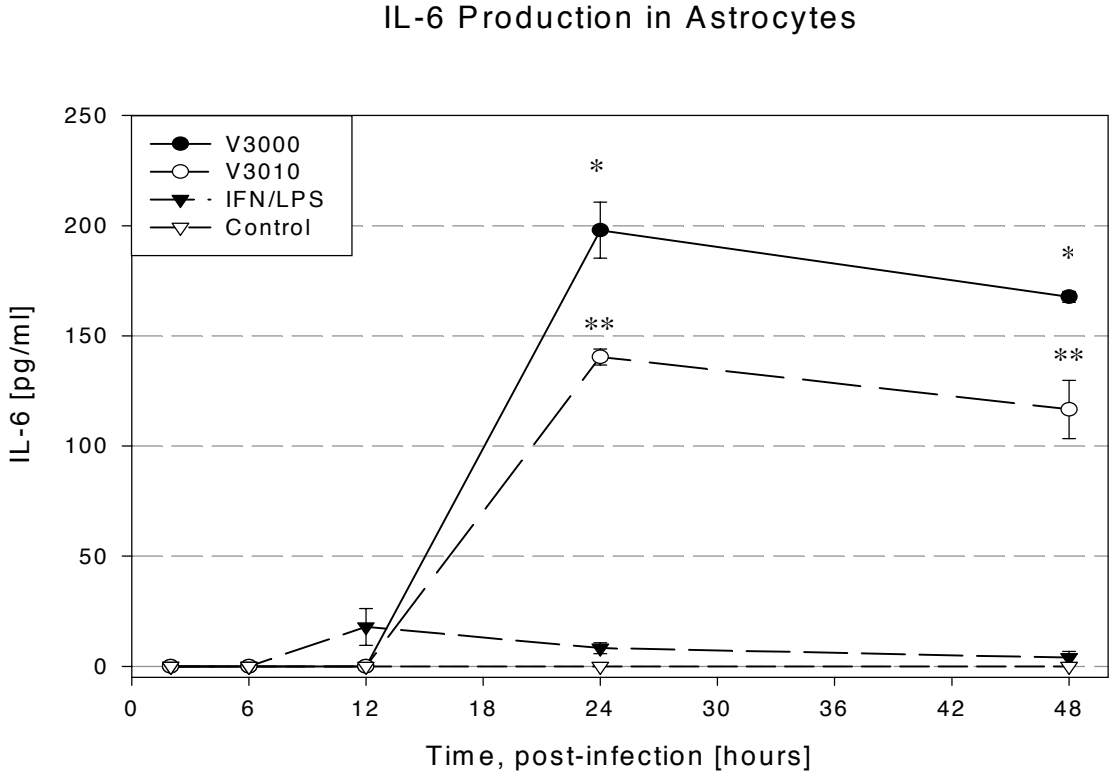
Southern blots of RT-PCR products for gene expression of IL-6. Primary astrocytes were infected with virulent V3000 or neuro-attenuated V3010 at a multiplicity of infection of 1.0. Both strains of VEE demonstrate gene induction as early as 6 hours p.i.

Figure 2



Histograms (mean ± SEM of three independent samples) representing changes in gene expression as determined by Southern blot analysis for IL-6 based on optical density measurements. Both virulent V3000 and attenuated V3010 induced IL-6 gene expression in primary astrocytes as compared to uninfected controls (*$p < 0.05$).

Figure 3



IL-6 protein was measured by ELISA in the supernatants of primary astrocyte cultures following infection with virulent V3000 or neuro-attenuated V3010. Values expressed as means ± SEM from three independent samples. Infection of astrocytes with both VEE strains resulted in IL-6 secreted in the culture supernatant at 24 and 48 hrs p.i. ($*p < 0.05$), however, neuro-attenuated V3010 infection of astrocytes resulted in lower IL-6 levels as compared to virulent V3000 at both 24 and 48 hrs p.i. ($**p < 0.05$). IFN-γ/LPS-stimulation of astrocytes resulted in low levels of IL-6 secretion at 12 hrs, but these levels declined and returned to near baseline levels by 48 hrs.

Figure 4. RNase Protection Assay for neurotrophic factors. Adult six to eight-week-old C57BL/6J mice were mock-infected or infected with 1 x 10^3 PFU of virulent V3000. Brain hemisections were harvested at 3, 4, 5, 6, and 7 days p.i., and total RNA was extracted and analyzed. Qualitative analysis demonstrates that neurotrophic support in the CNS was not different in V3000-infected mice when compared to mock-infected control mice throughout the 5-day experimental time course. Controls include undigested and RNase-digested (^{32}P)-labeled riboprobes.

Figure 4

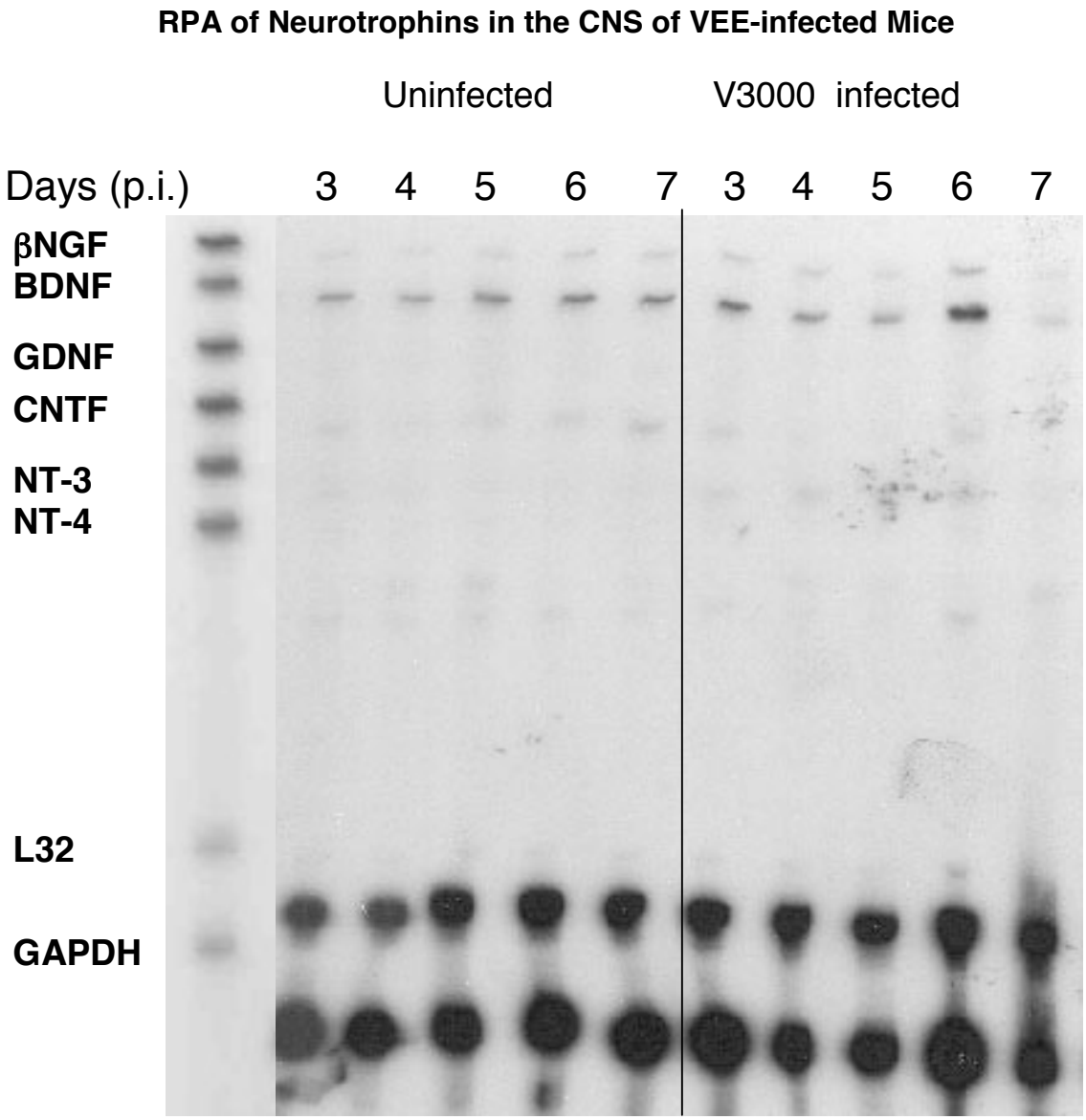
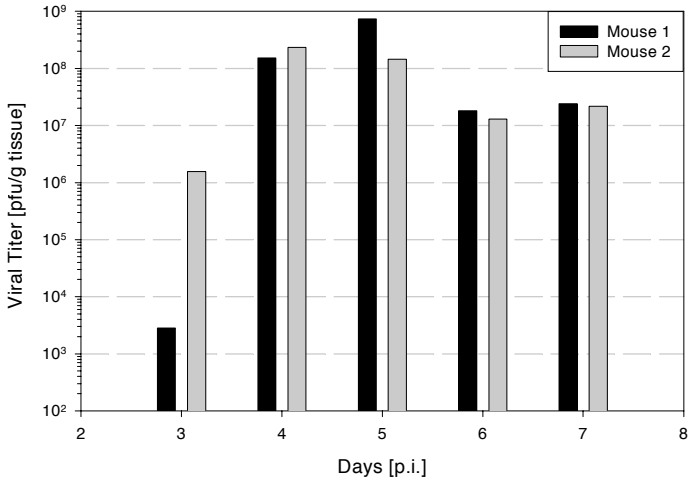

RPA of Neurotrophins in the CNS of VEE-infected Mice
Uninfected
V3000 infected
Days (p.i.)
3 4 5 6 7 3 4 5 6 7
βNGF
BDNF
GDNF
CNTF
NT-3
NT-4
L32
GAPDH

Figure 5. Histograms of VEE titers (PFU/g tissue). Six to eight-week-old female C57BL/6J mice were inoculated via left rear footpad injection with V3000, V3010, or V3034 at 1 x 10^3 PFU/animal. Two mice (black and gray bars) were sacrificed at each time point, and brains were removed for virus titration by standard plaque assay (n = 10 mice per group). Virus detection level was at 1.65 x 10^2 PFU/g tissue. Histograms demonstrate that VEE has established infection in the CNS at day 3 p.i., regardless of phenotype, and that this infection persists throughout the experimental time course.

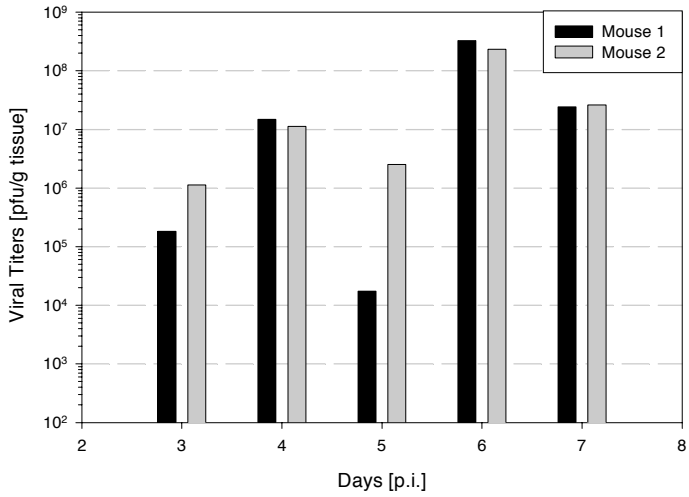
Figure 5

Viral Titers in the CNS of VEE-infected Mice

V3000



V3010



V3034

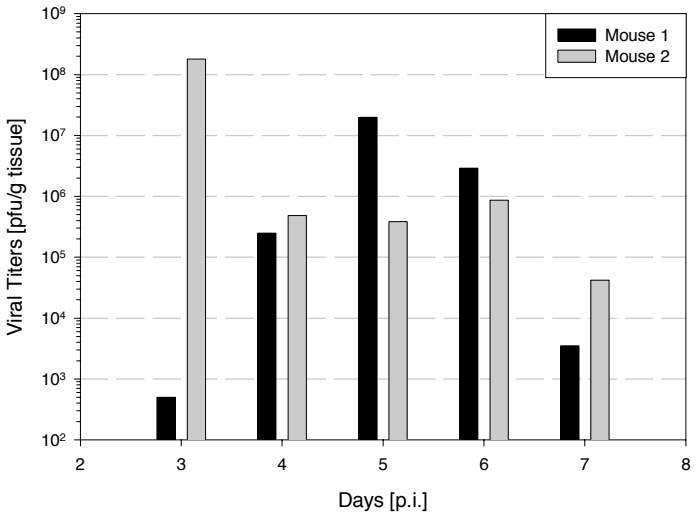
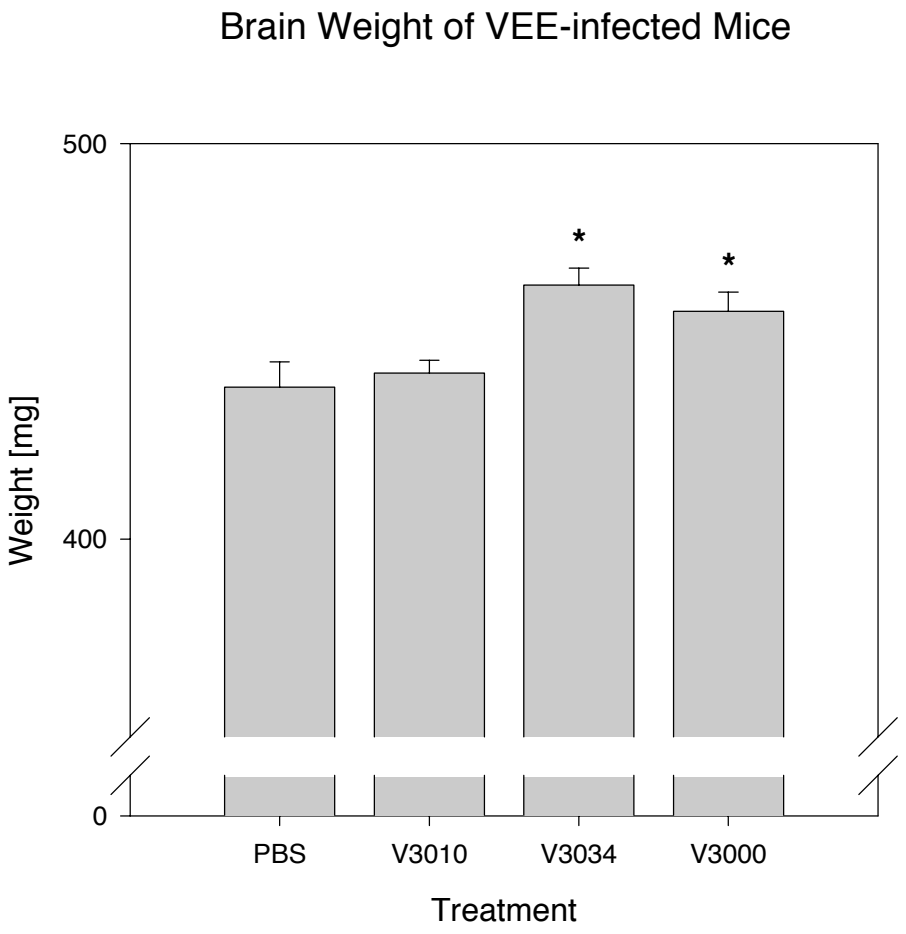
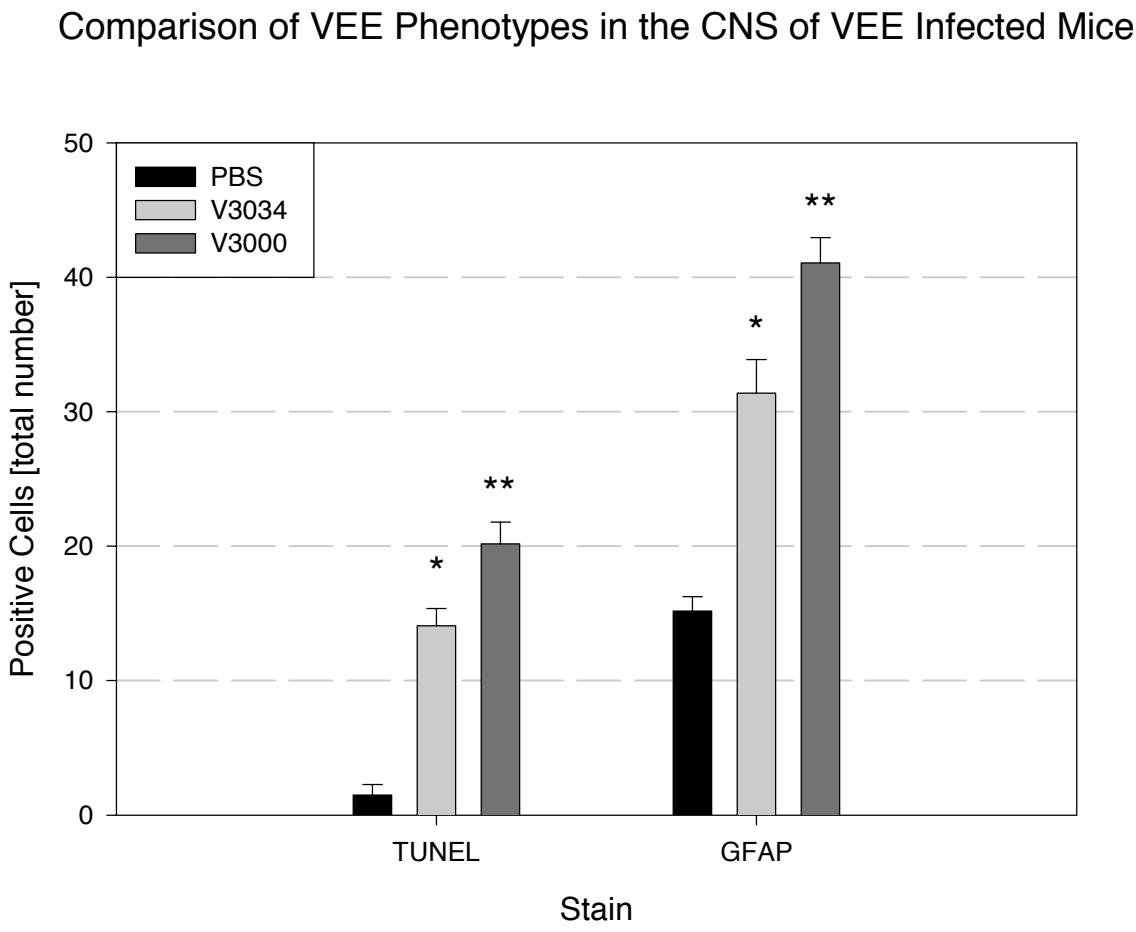


Figure 6



Age and sex-matched C57BL/6J mice were randomly assigned to one of four treatment groups (n = 10 mice per group) and weighed to assure homogeneous grouping of animals. Animals were infected via left rear footpad injection with 1 x 10^3 PFU of V3000, V3010 or V3034 or diluent. Mice were sacrificed at day 7 p.i. and brains harvested to include bilateral olfactory bulbs, cerebrums, cerebellums, diencephalons, and brain stems. Brain weights were determined on a calibrated precision scale and expressed as means ± SEM. Both V3000 and V3034 had significantly increased brain weights as compared to uninfected controls (*$p < 0.05$) indicating that these mice had cerebral edema.

Figure 7



Age and sex-matched C57BL/6 mice were infected with 1 x 10^3 PFU of either virulentV3000 or neuro-attenuated V3034, or mock-infected (n = 3 mice per treatment group). CNS tissue was harvested at day 7 p.i., processed and double-labeled for astrocytes and apoptosis. Total number of positive staining cells (red, astrocytes; black, apoptosis) for each treatment group were counted in high power fields from six different regions in the CNS (lateral cortex, interior cortex, thalamus, hippocampus, cerebellum, and brain stem). Values are expressed as means ± SEM. Both V3000 and V3034 were significantly different (*$p < 0.05$) as compared to uninfected controls, and V3000 had significantly increased staining for both astrocytes and apoptosis as compared to neuro-attenuated V3034 (**$p < 0.05$).

CHAPTER 5

Discussion

This investigation determined that astrocytes are permissive for to VEE infection *in vitro* and that there were virus strain differences in replication kinetics between virulent and attenuated VEE. Specifically, virulent VEE replicated to higher titers as measured in astrocyte culture supernatant at earlier time points p.i. There also appeared to be a trend in differences in the rate of astrocyte cell death comparing virulent and attenuated VEE strains, and the consequence of VEE infection in astrocytes is cytolysis. In addition, our experimental findings demonstrated that there were morphological features of VEE-infected astrocytes that suggest a subset of these cells may be undergoing apoptosis. These features include chromatin condensation and fragmentation, and the formation of apoptotic bodies as observed by light microscopy. Further, astrocytes respond to VEE infection by the upregulation of pro-inflammatory genes including TNF-α, iNOS, and IL-6. We also demonstrated that mRNA of TNF-α, iNOS, and IL-6 was translated into functional proteins as measured by enzyme-linked immunosorbent assay (ELISA) or Griess reaction analyses and that the amount of protein produced consistently depended on the VEE phenotype. Astrocytes cultures infected with the virulent V3000 VEE strain produced more TNF-α, $^{\bullet}$NO, and IL-6 as compared to the neuro-attenuated V3010 when measured by semi-quantitative RT-PCR and direct or indirect protein assays. There appeared to be a correlation between virus phenotype, virus replication, levels of cytolysis, and the early immune response produced by astrocytes *in vitro*. Attenuated V3010 had a significantly slower replication rate as

compared to V3000, as was the rate of cell death inV3010-infected astrocytes. Further, the innate immune response characteristics were significantly reduced.

VEE infection studies *in vivo* also demonstrated that there were significant differences in the immune response profiles of pro-inflammatory genes when comparing virulent and attenuated VEE phenotypes. Brain tissues of mice infected with virulent V3000 by peripheral footpad injection demonstrated a pro-inflammatory upregulation at 3 days p.i. and that this response was maintained throughout the experimental time course of 5 days. The response in mice infected with one of the attenuated VEE mutants depended on the particular virus strain. V3034, which has a single site amino acid mutation in the *E1* glycoprotein and a mortality rate of 20%, had a delayed induction of pro-inflammatory genes occurring at day 5 or 6 p.i. In contrast, V3010, which has a single site amino acid mutation in the *E2* glycoprotein and a mortality rate of 10% after footpad infection, did not appear to induce an inflammatory response when compared to uninfected controls. These responses were paralleled by the induction of pro-apoptotic genes in a pattern similar to those seen for the pro-inflammatory genes following VEE infection. Specifically, there were robust responses following infection with V3000 at 3 days p.i., delayed responses after infection with V3034, and undetectable responses following infection with V3010 as compared to uninfected control mouse brains.

Immunostaining of CNS tissue sections for VEE at 7 days p.i. and double-staining of adjacent tissue sections for astrocytes and apoptosis further contributed to the characterization of the early immune response to VEE infection. VEE infection in the CNS was randomly distributed in every area of the brain analyzed. However, this was not a panencephalopathic infection, in that VEE-positive antigen appear as specific foci.

This finding of widespread distribution of VEE antigen in the CNS in discrete focal lesions corroborates earlier work with VEE that demonstrated similar patterns of VEE antigen in the CNS in monkeys (Gleiser *et al*., 1962).

The pattern of astrocytosis and apoptosis was much more widely distributed in the CNS, and not limited to the focal areas of VEE infection. Further, double-labeling for astrocytes and apoptosis *in vivo* exhibited very few double-labeled cells. Together, these findings indicate that 1) astrocytes do not undergo apoptosis *in vivo* in response to VEE infection, 2) astrocytes increase in number or alternatively, increase in the intensity of GFAP expression in VEE-infected CNS tissue, 3) there is an association of astrocytosis with areas of apoptosis in the CNS, regardless of whether VEE antigen was present, and 4) the amount of astrocytosis and apoptosis in the CNS was significantly different comparing virulent and attenuated VEE strains. These results suggest that astrogliosis contributes to neurodegeneration by apoptosis. This finding is in contrast to the *in vitro* findings of morphological features of apoptosis in VEE-infected astrocytes. Although *in vitro* models are valuable in isolating specific cell types and characterizing their functional responses to experimental stimuli, there has been significant controversy, especially in the area of astrocyte research, as to the correlation of *in vitro* findings to those responses *in vivo* (Kimelberg, 1999). Certainly, astrocyte cultures have been valuable in characterizing functional immune roles, however parallel studies *in vivo* can be difficult to interpret or contrast with *in vitro* findings. Our contrasting findings of astrocyte apoptosis *in vitro* and the absence of astrocyte apoptosis *in vivo*, demonstrate that there are many extraneous variables *in vivo* that are excluded *in vitro*.

The results from the *in vivo* studies are the first steps in the characterization of the innate immune responses in the CNS to VEE infection. Pro-inflammatory and pro-apoptotic gene expression profiles have been characterized for three VEE phenotypes, the virulent V3000, and the two attenuated strains V3034 and V3010, over a 5 day experimental time course (3 - 5 days p.i.). This time course was selected because it includes the time when all three phenotypes have established infection in the CNS (Grieder *et al*., 1995) until the mean day of death for the virulent V3000. The gene expression characteristics are significantly different, depending on the VEE phenotype used for infection by peripheral footpad injection in mice. To investigate whether astrocytes undergo apoptosis in response to VEE infection *in vivo*, CNS tissue from VEE-infected adult mice were analyzed at day 7 p.i. Day 7 post-infection was investigated because mice infected with the virulent V3000 for this duration have severe encephalitis and are close to death. Therefore, it was hypothesized that this time point would demonstrate the most pathologic changes. Indeed, significant apoptosis was demonstrated in the CNS at day 7 p.i., and the amount of apoptosis was dependent on the VEE phenotype. However, other possible explanations could account for the differences in apoptosis observed following infection with the virulent *versus* the attenuated VEE strain. One such possible explanation is that apoptosis may occur at different times p.i., depending on the VEE phenotype. An experimental time course study using the double-labeling technique for astrocytes and apoptosis would help elucidate whether apoptosis occurs at different times depending on the VEE phenotype. This information could be used to obtain a better correlation between apoptosis, neurodegeneration, and the expression of pro-inflammatory and pro-apoptotic genes.

Other experimental models could also be expanded to test the hypothesis of specific cytokines and the development of neuropathogenesis. Specifically, the knockout mice studies described in this dissertation suggest that iNOS and TNF-α are components of the pro-inflammatory response that are likely to contribute to the demise of the VEE-infected host. Furthermore, it is possible that iNOS and TNF-α play a synergistic role in the pro-inflammatory response that results in neurodegeneration. Immunomodulation studies utilizing iNOS knockout mice along with TNF-α inhibition by antibody administration following VEE infection would help elicit whether iNOS and TNF-α are synergistic in their effect by measuring mortality rates. Previous studies have utilized anti-TNF-α to modulate the effect of TNF-α in mice against *Mycobacterium tuberculosis* infection (Flynn *et al*., 1995). However, there are no documented studies testing the synergistic effect or absence of iNOS and TNF-α in any infectious disease model.

Testing the hypothesis of iNOS and TNF-α synergism in the development of neurodegeneration with a complimentary experiment using TNF-α knockout mice and antibody administration against iNOS would be difficult because of inherent problems with the experimental design. It is unclear whether antibodies directed against iNOS would be effective due to the intracellular location of iNOS. In addition, its enzymatic product, $^{\bullet}$NO, is a diffusible free radical that makes antibody production against it impossible. However, there are iNOS inhibitors that could be administered to block the synthesis of $^{\bullet}$NO. Specifically, *N*-nitro-L-arginine methyl ester (L-NAME), an arginine analog that competitively blocks the conversion of arginine and molecular oxygen to $^{\bullet}$NO by iNOS, has been used to test the effect of $^{\bullet}$NO on viral replication and virulence of a flavivirus, *Japanese encephalitis virus* (Lin *et al*., 1997), demonstrating that $^{\bullet}$NO is host-

protective. In contrast, studies utilizing iNOS inhibitors testing viral replication and virulence of another flavivirus, *Tick-borne encephalitis virus* suggest that •NO may actually contribute to the pathogenesis of this virus infection (Kreil and Eibl, 1996). In the VEE-murine model system, an experiment comparing mortality rates between TNF-α knockout mice and control mice with the administration of L-NAME would help substantiate whether TNF-α and •NO act synergistically in the pathogenesis of VEE infection. In parallel to the mortality experiments, mice infected with VEE in identical groups would be sacrificed and brain samples collected to determine the experimental effects on virus replication, the induction of pro-inflammatory and pro-apoptotic genes, and for histology and double-labeling to assess for astrogliosis and apoptosis. Results from these studies would significantly contribute to our understanding of the multi-factorial effects of inflammatory-mediated neurodegeneration.

One other alternative to the proposed experiments to modulate TNF-α and iNOS to test for synergistic activity would be to breed TNF-α and iNOS knockout mice to produce a homozygotic double-knockout mouse strain. Progeny mice, at least two generations from the original parents, could then be genotyped to determine TNF-α/iNOS double-knockout phenotypic pups. These double-knockout pups could then be utilized to establish a breeding colony to produce enough mice in substantial numbers to conduct experiments. The viability of a TNF-α/iNOS double-knockout mouse strain is unknown.

Future studies investigating neurodegeneration in VEE infection should also address the role of other glial cells, in the early immune response, specifically microglia. These resident macrophage-like cells of the CNS may contribute significantly to the

production of biologically active substances that have important antiviral effects, but may also contribute to neurodegeneration. Previous studies characterizing replication rates of VEE in unstimulated or IFN-γ-stimulated primary microglia or macrophage cultures demonstrated significant reductions in VEE replication rates in IFN-γ-treated microglia or macrophages (Schoneboom *et al*., 2000b, Grieder and Nguyen, 1996). This finding of VEE replication inhibition in activated microglia *in vitro* suggests that microglia may play a central role in the antiviral defenses against VEE.

Other questions that deserve investigation are the sex and strain differences in the murine model. Most *in vivo* investigations of VEE pathogenesis use female mice. The reasons for using female mice are two-fold: 1) using age and sex-matched animals minimizes extraneous variability that can be introduced into experimental designs and, 2) the husbandry of female mice housed in a confined space of a bio-safety containment facility is easier. However, the exclusion of male animals may also eliminate important discoveries. Investigations comparing viral infections in the CNS between male and female mice using aerosolized *Vesicular stomatitis virus* (VSV) found striking differences in virus replication rates, spread of the virus from rostral to caudal locations in the CNS, and animal recovery times demonstrating a significant sexual dimorphism in the CNS against VSV (Barna *et al*., 1996). In addition to sex differences, are issues related to mouse strain differences in immunological models. Recently, phenotypic differences have been discovered between genetic backgrounds of inbred strains that are used to make target mutations for the generation of knockout mice (Anthony *et al*., 1988, Threadgill *et al*., 1995, Huang, 1999, Doetschman, 1999, Zhang *et al*., 1997, Erickson, 1996, Weichman and Chaillet, 1997, Corcoran and Metcalf, 1999, Schoneboom and

Grieder, unpublished data). Understanding these phenotypic differences in inbred mouse strains in response to VEE infection would be important in isolating critical components of an early immune response. Specifically, C57BL/6J and 129Sv/Ev mice are two inbred mouse strains that have been used frequently in the generation of knockout mice and have been used in research investigations with VEE (Grieder and Vogel, 1999, Schoneboom *et al*., 2000b, Schoneboom *et al*., 2000a). Understanding differences in immunological responses between these two mouse strains in response to VEE infection in the CNS would be beneficial in the interpretation of experimental data from the literature and further the understanding of molecular and cellular mechanisms underlying these responses.

In conclusion, the results from my investigations are the first steps toward delineating the innate immune response in the CNS in response to VEE infection. Specifically, these results demonstrate that there were differences in immune responses to virulent and attenuated strains of VEE, both *in vitro* and *in vivo*. Infection with virulent VEE induced a stronger, more robust up-regulation of several pro-inflammatory genes including iNOS, TNF-α, and IL-6, as compared to the two attenuated VEE strains. Furthermore, these responses correlated with cell death, neurodegeneration, and mortality. In contrast, the immune response did not correlate to the viral burden in the CNS. Independent of the phenotype of VEE, virus titers in the CNS following peripheral infection were indistinguishable over a 5 day observation period. In addition, these results identify an important role for astrocytes, both *in vitro* and *in vivo*, as contributors to the early immune response to this encephalitic/neurotropic viral infection. Taken together, these findings contribute to our understanding of the inflammatory response to

viral CNS infections and to the delicate balance between host protection and neurodegeneration. A better understanding of this complex system of innate immune responses is essential to develop therapeutic treatment modalities that target and modulate specific immune responses in the CNS.

Appendix

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